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CIBA FOUNDATION COLLOQUIA ON AGEING

VOLUME 2

Ageing in Transient Tissues

Editors for the Ciba Foundation

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With 96 Illustrations



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PREFACE

THE Ciba Foundation, London, is an educational and scientific charity founded by a Trust Deed made in 1947. Its distinguished Trustees, who are wholly responsible for its administration, are The Rt. Hon. Lord Adrian, O.M., F.R.S.; The Rt. Hon. Lord Beveridge, K.C.B., F.B.A.; The Hon. Sir George Lloyd-Jacob; and Mr. Raymond Needham, Q.C. The financial support is provided by the world-wide chemical and pharmaceutical firm which has its headquarters in Basle, Switzerland.

The Ciba Foundation forms an international centre where workers active in medical and chemical research are encouraged to meet informally to exchange ideas and information. It was opened by Sir Henry Dale, O.M., F.R.S., in June 1949.

In the first six years, in addition to many part-day discussions, there have been 37 international symposia, each lasting two to four days, attended by outstanding workers from many countries. Other symposia are planned at the rate of five or six a year.

Early in 1954 the Trustees decided on special measures designed to encourage, internationally, basic research relevant to the problems of ageing. As part of this programme the conference already arranged in that year on General Aspects of Ageing was taken to be the first in a series of colloquia, to be held annually. A second colloquium was organised in 1955, and at the welcome suggestion of Professor E. C. Amoroso, this one dealt with studies on the ageing of tissues, the normal life of which is shorter than that of the organism as a whole. It was hoped that inferences of more general value might be made from researches on the ageing of the placenta, the reproductive system, deer antlers, erythrocytes and so on.

This volume contains papers and discussions of this second colloquium, on Ageing in Transient Tissues, at which Professor Amoroso graciously acted as Chairman.

The informality and intimacy of these meetings have permitted discussion of current and incomplete research and stimulated lively speculation and argument. They have also been the occasion for reference to much published and unpublished work throughout the world. The proceedings are issued in full, with only the minimum of editing, in order to pass on to a far wider audience the benefits of these meetings. It is hoped that readers will not only gain information and inspiration from this report, but will also feel that they share in these frank and friendly discussions.

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5th–7th July, 1955

E. C. Amoroso	Royal Veterinary College, London
F. Bourlière	Faculté de Médicine de Paris
J. D. Boyd	Anatomy Dept., Cambridge University
G. W. Corner	Embryology Dept., Carnegie Institution of Washington
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G. S. Dawes	Nuffield Inst. for Medical Research, Oxford
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R. J. Harrison	Anatomy Dept., London Hospital Medical College
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P. L. Krohn	Dept. of Anatomy, University of Birmingham
L. Harrison Matthews	Zoological Society of London
P. B. Medawar	Zoology Dept., University College, London
P. L. Mollison	Postgraduate Medical School of London
W. Montagna	Biology Dept., Brown University, R.I.
A. S. Parkes	National Institute for Medical Research, London
Dorothy Price	Zoology Dept., University of Chicago
I. W. Rowlands	Institute of Animal Physiology, Brabaham, Cambridge
F. Strauss	Anatomy Dept., University of Berne
H. Tuchmann-Duplessis	Faculté de Médicine, Université de Paris
C. A. Villee	Biological Chemistry Department, Harvard University Medical School
P. C. Williams	Imperial Cancer Research Fund, London
G. B. Wislocki	Anatomy Department, Harvard University Medical School
E. W. Yemm	Dept. of Botany, University of Bristol
S. Zuckerman	Anatomy Dept., University of Birmingham

CHAIRMAN'S OPENING REMARKS

E. C. Amoroso

I am deeply conscious of the honour which is mine in being entrusted this morning with the chairmanship of this Symposium, the thirty-fourth I believe, in the CIBA Foundation series and the second on the problems of ageing. When about a year ago the ageing of transient tissues was suggested by Dr. Parkes as a possible subject for this Symposium and having no alternative of my own to offer, I accepted the suggestion with a procrastinator's easy optimism. Only during the past week, when I received the complete programme from Dr. Wolstenholme, did I begin to recognize the immensity of the problem; and if I venture upon the task to-day of presiding at this meeting, it is not because I have any special knowledge, but only because I realise that among this audience there are a number of experts from both sides of the Atlantic—many of them friends of long standing—whose function it will be to provide a picture of the present status of the subject, which it is our business to discuss. My task will be, to offer, at a somewhat later stage of the proceedings, some kind of general synthesis combining the diverse views—which I imagine will be frequent at this meeting—and not to attempt a speculative review at this stage of what the succeeding papers will most certainly present in great detail.

For any shortcomings that may become apparent in the conduct of the business of this meeting which will take us certainly very far beyond the confines of the problem of ageing such as we normally concede it to be, I must ask for your indulgence, and I hope I may be permitted the same latitude which so many of the Symposiasts have taken with regard to the interpretation of the title.

The subject we are to discuss comprehends a great variety of problems, ranging from the physical instability and ageing of red blood cells to the metabolism of senescent leaves. This meeting, it is hoped, should help in combining these diverse points of view. And so it gives me great pleasure to call on Dr. Dorothy Price to present the first paper.

ORGAN CULTURE STUDIES OF FOETAL RAT REPRODUCTIVE TRACTS

Dorothy Price and Richard Pannabecker
Zoology Department, University of Chicago

In rodents, the question of the rôle of endogenous sex hormones in the retention or loss of Wolffian ducts and Müllerian ducts and in the formation of the primordia of accessory reproductive glands has not been completely answered.* The problem has been investigated chiefly by 1) administration of sex hormones to pregnant females (the most extensive researches are those of Raynaud, 1942; Greene and his collaborators, reviewed by Greene, 1942); 2) transplantation of foetal reproductive tracts into postnatal hosts (Moore and Price, 1942); 3) foetal gonadectomy by X-ray (Raynaud and Frilley, 1946, 1947, 1950; Raynaud, 1950) and by surgical castration (Wells and Fralick, 1951; Wells, Cavanaugh and Maxwell, 1954).

Organ culture offered a technique for further analysis of the problem by isolation of foetal reproductive tracts from other foetal endocrine organs in an environment free from maternal and placental hormones, and under conditions in which the gonads could be removed with a minimum of traumatic injury. It was recognized that, if sex hormones were present in the testes or ovaries, diffusion might be expected since this has been demonstrated for foetal rat testes (Jost, 1948; Jost and Colonge, 1949; Moore, 1953).

Methods

The methods will be described in detail elsewhere (Pannabecker, 1956). In brief, about three hundred foetal rat

* Many of the problems of sex differentiation in the rabbit, a member of the Lagomorpha, have been clarified by the research of Prof. Jost.

reproductive tracts (equal numbers of male and female) were cultured by the watch-glass method (Fell, 1940) at approximate ages of 15·5, 16·5, 17·5 and 18·5 days post-copulation. The culture period ranged from one to six days with transfer on the second or third day. The tracts were dissected under sterile conditions; the bladder, genital tubercle and, in many cases, the posterior urogenital sinus and urethra were removed. In some categories of explants, one or both testes were also removed by severing the efferent ducts and freeing the testes without cutting the Wolffian ducts. The tracts were then placed on large clots composed of cock plasma and chick embryo extract. This medium had been tested and found to have no stimulating effects on the differentiation of prostate glands explanted from six-day-old rats (Price, 1951, 1953). Some of the foetal explants were subjected to testosterone or oestradiol, which was added to the clots in the form of micro-pellets in saline suspension. The explants were observed daily and the course of persistence or retrogression of the ducts was followed. At termination of the culture period the explants were fixed and studied. This report will be limited to the results on male Wolffian ducts, seminal vesicles and prostate glands.

The male tracts were explanted in the following categories:

1) with both testes
2) with one testis
3) with one testis which had been detached and replaced in position (the second testis was removed)
4) with no testes
5) with ovaries replacing the testes
6) with no gonads; testosterone in the clot
7) with no gonads; oestradiol in the clot

Categories 2 and 3 tested whether there was local action from diffusing testis hormone. In addition, category 3 was especially designed to determine whether any observed retrogression of Wolffian ducts after testis removal was attributable to surgical injury or to absence of the testis.

Normal Development of the Wolffian Ducts, Seminal Vesicles and Prostate Glands

In the foetus at 15+ days the Wolffian ducts are continuous from the efferent ducts to the urogenital sinus. During the following day there is an increase in the diameter of the ducts in the genital cord, and by 17+ days there is a definite dilatation in each Wolffian duct (Fig. 2, A) in the region where, by 18+ days, a seminal vesicle primordium develops as a dorsal outgrowth with a free tip, which continues to grow anteriorly (Fig. 2, D and F) and forms a cranial flexure. The Wolffian duct differentiates before birth into the coiled epididymis, the ductus deferens and the ejaculatory duct opening into the prostatic urethra.

The prostate glands develop as dorsal, lateral and ventral buds from the prostatic urethra and the coagulating glands originate as paired buds from the dorsal region. A few prostatic buds may be present at 18+ days but the primordia of all the lobes of the prostatic complex and the coagulating glands are developed by 19+ days (Fig. 3, A).

Results

The explanted reproductive tracts did not increase in length but under certain conditions they continued to differentiate at a somewhat slowed rate. The results from culture of the youngest explants were somewhat variable and necrosis occurred more frequently, particularly in tracts explanted at 15+ days. However, the results were clear at all ages and the older tracts survived very well in culture.

Wolffian Ducts

In 15+ day-old tracts that were explanted with testes (both, one, or one that had been detached and replaced) the Wolffian ducts persisted as continuous ducts for the longest culture period of 5 days (Table 1). The two ducts were about equally well maintained when only one testis was present, and the

Table I

DEVELOPMENT OF WOLFFIAN DUCTS, SEMINAL VESICLES AND PROSTATE GLANDS IN CULTURED REPRODUCTIVE TRACTS

Explants				*Wolffian ducts*	*Seminal vesicles no.*	*Urogenital Sinus no.*	*Prostate glands no.*
Categories	*Age in days post-coitum at*		*total no.*				
	explantation	*termination*					
with testes	15+	17+ – 20+	14	persisted	0	8	1 (19+)
	16+	20+ – 22+	24	"	9 (20+)	16	14 (20+)
	17+	19+ – 21+	10	"	18 (19+)	13	12 (19+)
	18+	21+	6	"	6	0	
no gonads	15+	17+ – 18+	4	regressed	0	4	0
	16+	19+ – 20+	9	"	0	5	4 (20+)
	17+	19+ – 21+	7	"	0	6	5 (20+)
	18+	20+ – 22+	4	"	4	2	2 (21+)
with ovaries	15+	16+ – 18+	4	regressed	0	3	0
	16+ –	19+	2	"	0	2	0
	17+	19+ – 21+	4	"	0	2	2 (20+)
	18+	21+ – 22+	3	"	3	2	2 (21+)
testosterone	15+	16+ – 18+	9	persisted	1 (18+)	3	0
	16+	20+	4	"	2 (20+)	4	4 (20+)
	17+	19+ – 22+	6	"	6 (19+)	5	4 (19+)
oestradiol	15+	17+ – 10+	3	regressed	0	3	0
	16+	19+ – 20+	4	partial persistance	2 (20+)	2	0
	17+	20+ – 21+	7	"	2 (20+)	6	5 (20+)
	18+	21+ – 22+	4	"	4 (21+)	4	4 (21+)

The urogenital sinus was removed at explantation in some cases.

Seminal vesicles were present in explants cultured at 18+ days; some small prostatic buds occur in a few foetuses at 18+ days but are normally present by 19+.

The figures in parentheses indicate the age of the youngest explant in which seminal vesicles or prostate glands were found.

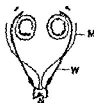
results were similar with a replaced testis and one left attached indicating that surgical removal of the testis did not cause Wolffian duct retrogression.

In contrast, in tracts with no testes the Wolffian ducts underwent slow retrogression which involved successively, reduction in diameter, loss of the lumen and then of the epithelial component so that the ducts were discontinuous, and finally loss of the surrounding sheath of mesenchymal origin. Retrogression was mainly antero-posterior and by 3 to 4 days of culture the ducts were practically gone anteriorly, except for a small segment connected with the efferent ducts; the posterior segment persisted and joined the prostatic urethra. Testosterone in the clots (1 or 2 drops of 4μg./drop or 20μg./drop) prevented retrogression of the ducts after removal of the testes.

The results of culturing explants at the three older ages, with both testes on the tracts or without testes, were essentially similar to the results with 15+ day-old explants. With both testes present the Wolffian ducts persisted and developed a slight epididymal coiling; without testes the ducts retrogressed. An important difference however, was observed in tracts with only one testis (left in position or replaced). Although both Wolffian ducts usually persisted, there was a unilateral effect of the one testis if the tracts were spread in an open Y-shape on the clots. It was apparent that the diameter, and in some cases the continuity, of the gonadless Wolffian duct depended upon its distance from the testis. Fig. 1 presents the results diagrammatically for explants cultured at 17+ days.

In the gonadless tracts cultured at all four ages, testosterone prevented retrogression of the Wolffian ducts. When ovaries were substituted for testes the ducts retrogressed as in gonadless explants, and the same result was obtained in tracts cultured at 15+ days with oestradiol. At older explantation ages, oestradiol (1 or 2 drops of 0·006μg./drop in the clots) caused a partial retention and some cystic enlargement of the ducts in 5 out of 15 explants.

DAYS
POST COITUM
17.5



21.5











FIG. 1. Diagrammatic representation of reproductive tracts explanted at 17+ days. A: tract at the time of explantation; B, C, D, E, F: tracts cultured for 4 days. B: with both testes present the seminal vesicles and prostate glands developed. C: without testes the Wolffian ducts retrogressed, no seminal vesicles appeared but a few prostatic buds developed. D: with one testis (left in place or detached and replaced) the results were as in B in 5 out of 6 explants. E: with one testis, placed at a greater distance from the opposite side of the tract, the Wolffian duct on the gonadless side retrogressed somewhat and the seminal vesicle was lacking or smaller in 8 out of 9 explants. F: with no testes but with testosterone micropellets added to the clot, the Wolffian ducts were retained and the seminal vesicles and prostatic buds developed as in B and D.

M: Müllerian duct; W: Wolffian duct; S: seminal vesicles; P: prostate.

Seminal Vesicles

The development of seminal vesicles proved to be related to the age of the tract at the time of explantation as well as to the presence of hormone. In explants with testes, no seminal vesicles developed in tracts cultured at 15+ days; large dilatations with only a slight indication of a free seminal vesicle tip appeared in 9 out of 24 explants cultured at 16+ days; well-developed glands were found in 18 out of 19 explants of 17+ day-old tracts cultured when dilatations were present in the Wolffian ducts (Fig. 2, A), and within four days of culture they reached a length, in the best cases, equal to

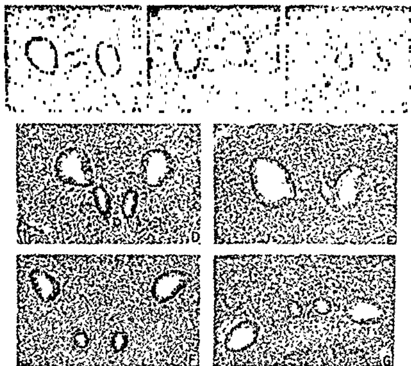
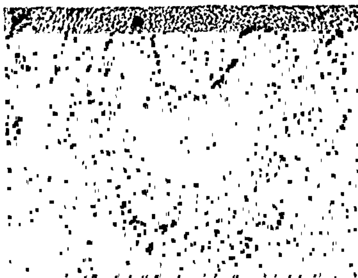
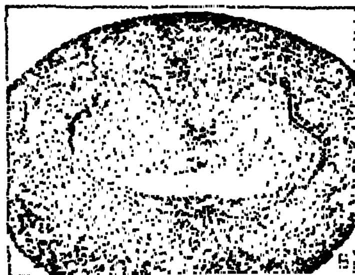


FIG. 2. A, D and F illustrate normal foetal development of the seminal vesicles; B, C, E and G were explanted at 17½ days and were cultured for

ejaculatory duct on the left, ductus deferens and seminal vesicle on the right. F: normal foetus at 21+ days; length of the seminal vesicles 1·1 mm. G: 21+ day-old explant with testes; length of the seminal vesicles 0·16 mm. (× 90).





that of normal glands at about 19 days (Fig. 2, B, D, G) but no cranial flexures developed. Seminal vesicles were already developed in tracts explanted at 18+ days.

A unilateral effect of one testis on seminal vesicle development was observed. In 26 such explants in which seminal vesicles developed, the glands were larger or present only on the testis side in 20 but were bilateral and equally developed in 5 explants in which the testis was near the gonadless Wolffian duct (Fig. 1).

In explants with no testes or with ovaries, no seminal vesicles developed (16+ and 17+ days) or they remained as at explantation (18+ days). Testosterone stimulated the development of seminal vesicles and was more effective with advancing age of the tract at the time of explantation. As in explants with testes, only the tracts explanted at 17+ days were stimulated to produce well-developed seminal vesicles (Fig. 2, E). Oestradiol stimulated seminal vesicle development in 4 out of 11 explants cultured at 16+ and 17+ days.

Prostate Glands

Part of the urogenital sinus region and the urethra were removed from some reproductive tracts at explantation and when the sinus was explanted, it tended to undergo necrosis particularly in the region of Müller's tubercle. Table I lists the number of well-preserved sinuses and summarizes the occurrence of prostate glands.

The prostatic buds that developed were from the dorsal and dorsolateral regions and probably represented those parts of the prostate gland complex and possibly the coagulating glands (Fig. 3, B). The removal of the bladder at explantation disturbed the ventral part of the sinus and urethra and, although healing occurred, no prostatic buds were formed from that region. Only one prostate gland (of one or two short buds) developed in explants cultured at 15+ days and that with testes on the tract. At older ages, prostate glands developed in all categories of explants. There was variability in

the number and size of the buds but, in general, the prostatic buds were more numerous and larger in explants with testes or with testosterone.

Discussion

The results of the culture experiments indicate that a hormone from the foetal testes maintained the Wolffian ducts, stimulated the formation of the primordia of the seminal vesicles and their growth, and caused an increase in the number of prostatic buds. These findings extend and corroborate some of the observations of Wells and his co-workers (Wells and Fralick 1951; Wells, Cavanaugh and Maxwell, 1954) in castrated rat foetuses. They concluded that testicular androgen stimulates the prenatal growth of male rat accessory reproductive organs but that they could not determine whether this androgen caused the development of the primordia of these glands. They also observed absence of the flexure of the seminal vesicles and loss of the epithelium of the ductus deferens following castration and attributed this to two factors, the absence of the testes and impairment of deferential circulation due to surgical injury (Wells and Fralick, 1951). Testosterone treatment, however, prevented all of the observed effects of castration. In unilateral castration they found effects on the ductus deferens and the seminal vesicle on the operated side in some foetuses, but they considered that these results were due to circulatory impairment and did not support the idea of local action of a diffusing testis hormone.

Explantation of foetal rat reproductive tracts at ages from 15·5 to 18·5 days gave results that suggest that the retention of the Wolffian ducts and the development of seminal vesicle primordia may normally depend upon testis hormone. Under culture conditions, this hormone reached other parts of the tract by diffusion and produced local effects on Wolffian ducts and seminal vesicles in unilaterally castrated explants. Further, the results of detaching and replacing testes on the

tracts indicate that surgical injury can be ruled out as a significant factor in general Wolffian duct retrogression.

The question of the relation of testis hormone to the development of the prostate glands cannot be definitely answered. Prostatic buds developed in gonadless tracts which had been explanted before the appearance of the primordia and this development was reported also for castrated foetuses (Wells, Cavanaugh and Maxwell, 1954). In culture, these buds formed in only one tract (with testes) which was explanted at 15+ days, but this result may represent only slowed development. It seems probable that the prostatic region of the urethra is subjected to the influence of testis hormone in the foetus as early as 15+ days, and it may soon be conditioned to produce a few prostatic buds even in the absence of further hormone stimulation. The hormone, as a diffusible agent, would act to bring out an underlying organization of the urethra. However, such an interpretation is complicated by the fact that prostate glands developed in foetal female tracts after explantation (Pannabecker, 1956).

The conclusions as to the significance of foetal rat testis hormone in relation to the Wolffian ducts, seminal vesicles and prostate glands and the observation of local action of testis hormone are in agreement with the findings of Jost (1947, 1950, 1953) in his extensive studies on castrated foetal rabbits. His results showed, however, that prostatic buds did not develop when castration was performed at the youngest ages. He and his collaborators (Jost and Bergerard, 1949; Jost and Bozic, 1951) reported the retrogression of the Wolffian ducts in cultured fragments of gonaducts of foetal rats. Raynaud and Frilley (1946, 1947, 1950) reported that following X-ray destruction of the testes in mice, the accessory reproductive glands were smaller or absent and a unilateral effect was observed when only one testis was destroyed.

The direct stimulating effects of oestradiol on the Wolffian ducts and on seminal vesicle development in a few gonadless explants is of interest, since it duplicates the results obtained in female rat foetuses when the mothers were injected with

oestradiol or oestradiol dipropionate (Greene, Burrill and Ivy, 1939, 1940). In the males, the results were variable and the Wolffian ducts were normal in some foetuses but had retrogressed in others; the seminal vesicles were smaller and prostatic buds were inhibited. These results suggest the possibility of an inhibiting effect of oestrogen on testis hormone production via the pathway of foetal hypophyseal gonadotrophin and, possibly, a direct stimulating action of oestrogen on the male tract. However, hypophysectomy by decapitation in the foetal rat has not shown that the hypophysis produces gonadotrophin, which is necessary for the secretion of testis hormone (Wells, 1947, 1950). Jost (1953, 1954) has reviewed the problem recently for several species.

Summary and Conclusions

The results of culture of foetal male reproductive tracts indicate that testis hormone 1) maintains the Wolffian ducts, 2) stimulates the development of the primordia of the seminal vesicles and their further growth and morphogenesis and 3) causes an increase in the number of prostatic buds. The testis hormone diffused through the explanted tracts and produced local effects.

It is suggested that these observations may apply to normal sex differentiation in the rat and that foetal testis hormone may be effective by local diffusion as well as by circulatory pathways.

Acknowledgement.

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DISCUSSION

Jost: I was very much interested in Dr. Price's magnificent results [illegible]

no Wolffian duct persists.

Some years ago, we tried in my laboratory to confirm by experiments *in vitro* the results obtained *in vivo* in castrated rabbit foetuses. I should like to show you two slides taken from former papers (Jost, 1950; Jost and Bozic, 1951). Small pieces of the genital tract containing both the [illegible]

gressed and disappeared while the Mullerian ducts developed and proliferated; so the ducts behave *in vitro* as they do *in vivo* in castrated rabbit foetuses. These results seem to be in good agreement with Dr. Price's more extensive experiments.

But, Dr. Price, you did not discuss the point of the Müllerian ducts.

Did you observe any inhibition of the Müllerian ducts in explants with the testes?

Price: I did not mention the Müllerian ducts because of lack of time. Our results on these ducts are still being studied and will be reported soon by Pannabecker.

We found that explanted Müllerian ducts of both males and females were more difficult to culture at 15½ and 16½ days than Wolffian ducts. They were actively growing and in a critical period of development at the time of explantation and they tended to become discontinuous under all our culture conditions. As I say, they had rather a hard time but they continued to develop and formed the prostatic utricle in males and the uterovaginal canal in females.

When we explanted older tracts, the Müllerian ducts of males regressed and the Müllerian ducts of females were retained under all experimental conditions. The Müllerian ducts of females were stimulated, not inhibited, when foetal testes were placed against them or

[illegible] ver, I do not think that this [illegible] foetal testis hormone may

[illegible] e prostatic development of tracts which were taken on day 16½ and those taken on day 17½? You showed that some prostatic buds appear, but the testis begins to work earlier than day 17½ and by that time the testis has been able to act on the urogenital sinus for about one day. I would also like to draw attention to the fact that in some of these cultures the effect of the distance from the testes was very conspicuous; I observed some years ago such a spatially restricted activity of the testis *in vivo* under certain circum-

[illegible]

I may say that in our cultures prostatic buds developed in explanted female tracts with ovaries present and without ovaries. Most of these

[illegible]

in spontaneous occurrence of female prostatic primordia, including unilateral lobes, on the basis of levels of foetal ovarian androgenic hormone.

[illegible]

interstitial cell situation is still being studied.

Corner: As I see it, the action of the foetal hormone in these cultures was accurately imitated by testosterone, and this suggests, as far as it goes, that the foetal hormone is similar chemically or identical with that of the mature testis.

Price: No, I do not believe that they are necessarily similar or identical.

Zuckerman: In your reply to Prof. Jost, you suggested that you might be dealing with a "prostatic strain" of female rats. Do most of the females show prostatic rudiments?

Price: Some years ago we established a stock of rats with an incidence [illegible]

Zuckerman: Could one infer that the presence of prostatic tubules in [illegible]

Price: I think that varying levels of androgenic hormone may not necessarily be the whole story in the development of prostatic primordia in females. [illegible]

or the adrenal gland may produce some androgenic compound?

Price: Yes. It is certainly true in postnatal rats, and prostate glands of males and females respond to such hormones.

Jost: In some cases, for instance in the mole, the ovary contains a very large medullary part which is like a testis, and in such an animal it seems

likely that the ovary may produce some androgenic compound; and indeed all the females of the mole are more or less masculinized. That is a special case.

Parkes: Prof. Jost, are you referring to the adult or the embryo in these moles?

Jost: Both. The mole embryo was studied by Godet (1949). He observed that the female foetus in the mole normally exhibits signs of

masculinized female of the rat, for instance.

Parkes: That is why I asked whether you were referring to the adult or the foetus.

Jost: It was done on the foetus.

Parkes: I imagine it can be taken as read that the adult ovary pro-

a seasonal cycle.

Parkes: You mean masculinization appears when the ovary has gone back to normal?

Amoroso: I think it should be emphasized that the morphological changes in the reproductive organs of the mole are so notoriously different from the comparable events in the rat that the greatest caution should be exercised when making comparisons between the two species.

But apart from this I would like to ask Dr. Price whether in fact the Wolffian ducts may not themselves exert a profound influence on the development of the Müllerian ducts. It is known, for example, that in elasmobranch fishes the Müllerian ducts arise by longitudinal splitting of the Wolffian ducts and in mammals there is good supporting evidence that the Wolffian ducts and/or Wolffian tubules contribute elements to the growing tips of the Müllerian ducts.

Price: Yes, I think there is a real point here. Gruenwald (1941) offers evidence for the chick and the human that the Wolffian duct may contribute cells to the Müllerian duct or alternatively that the Müllerian duct may split from the Wolffian duct.

In some of our explants we observed that the posterior end of a

age the ducts continued their growth to the sinus and joined it but growth was less marked in other explants. It is true that some of the variability may be attributable to morphological or physiological changes in the Wolffian ducts in culture.

Villee: Dr. Price, I am interested in your experiments in which the testis is replaced by synthetic hormones added to the culture, and I wonder if you can say anything about the quantity of hormone that is present in the clot?

In some cases, the oestradiol you added will have affected organs [illegible]

only about 1/1000th as active as oestradiol in stimulating the placental [illegible]

similar but quantitatively different?

Price: We put one or two drops of saline suspensions of micropellets in the clots. The testosterone suspensions were 4 μg./drop and 20 μg./drop and the oestradiol, 0·000 μg./drop.

With regard to the variability in the stimulating effects of oestradiol on Wolffian ducts I doubt that chance local distribution of the hormone is responsible. Greene and his collaborators found much the same variability in foetal females when large doses of oestrogens were injected into the pregnant mothers. I do not think that this is a significant finding as far as normal sex differentiation goes.

Villee: That would depend on whether these early organ anlage can produce hormones typical of the opposite sex.

Price: That is true.

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THE AGE FACTOR IN SOME PRENATAL ENDOCRINE EVENTS

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IT IS EVIDENT that age is an essential factor in the study of the foetal endocrine correlations, since the endocrines cannot be expected to play any physiological part before they reach an adequate stage of differentiation. However, physiological specialization does not necessarily parallel morphological organogenesis. *A priori* three points of view about the foetal endocrine glands may be considered and were effectively sustained: 1) the endocrines have no physiological importance at all for the developing foetus; such a view is now difficult to maintain, at least for some glands; 2) they progressively acquire a state of physiological activity which enables them to work immediately at birth; this interpretation often, and more or less implicitly, involves a similarity between prenatal and postnatal endocrine processes; 3) finally, one may assume that foetal endocrinology implies characteristic events occurring at certain stages of foetal life, and which can be distinct from postnatal function, even if they prepare postnatal life.

In connection with this last view it should be recalled that some organs pass through a limited phase of sensitivity to hormones. For instance, masculinization of the female urogenital sinus by testosterone can be produced only during a limited period of time (Turner, 1940; Moore, 1945). Moreover, hormonal treatments may become teratogenic if applied at certain stages: pitressin injected into the rat foetus produces haemorrhages, necrosis and, finally, congenital amputations of the extremities when administered before day nineteen and not afterwards (Jost, 1953c). In recent unpublished observations, I noticed that cortisone injected into the abdominal cavity of rat foetuses (1–3 mg. per foetus) produced cleft

palates only if given before day sixteen (this effect of cortisone was discovered on mice by Fraser and Fainstat, 1951).

In such cases the response of the foetal organism to the same treatment is strictly correlated with its developmental age. In the normal developing foetus, variations in the response of the same target organ to the same endocrine gland may also occur according to the age. The efficiency of the endocrine factor then becomes maximal at certain stages, and it seems not unlikely that the hormonal release might also reach a maximum at the same stages. Some facts presented in this paper will show to what extent such speculations may be suggested by experimental data; but no effort will be made to give a complete review of the field.

Testicular function and sexual structures

The testis is responsible for the development of the masculine sexual structures, as was observed in castration experiments on the rabbit foetus (Jost, 1947, 1953*a*); the importance of the age factor in these experiments will be recalled in connection with two structures.

The Wolffian, or mesonephric, ducts exist in both sexes at early stages; in females they persist until day twenty-four when they begin to regress; in males they develop as deferent ducts and seminal vesicles under the stimulation of the testis. They regress in males also, if castration is performed before day twenty-two; after castrating on day twenty-three only a small seminal vesicle develops, and after castrating on day twenty-four the Wolffian ducts persist and differentiate along their whole length. They have been "stabilized" for the remainder of their life; from transitory urinary ducts they have developed into definitive male genital ducts. Under the influence of the testis, something happens between about days twenty-two and twenty-four which changes the characteristics of the ducts and which enables them to differentiate as deferent duct and seminal vesicle, even in the absence of the testis. However, at that stage and still for a while, these ducts cannot

be stimulated to glandular activity as in the adult, even by large doses of testosterone.

The urogenital sinus provides another interesting example. This part is identical in young male and female embryos. Prostatic buds appear in the male if the testis has acted on the undifferentiated tissue for a sufficient period of time. Early castration, on day nineteen, completely prevents the formation of the buds; castration performed one or two days later permits the development of two straight unbranched buds; castrating two days later still, on day twenty-three, when only mere anlage have appeared, does not stop prostatic growth. The buds continue to differentiate, although generally in a somewhat reduced manner in comparison with control animals.

During the short period of time involved, the testis has completely changed the properties of a special part of the urogenital sinus, which has become *the prostatic region.* It would be of great importance to know what happened in these cells, what kind of change they underwent.

After day twenty-four the male rabbit foetus is definitely marked as a male; the sexual structures have passed a turning-point after which removal of the testis does not inhibit further differentiation. Even if the testicular function of the normal male were reduced at late stages of pregnancy, the sexual organogenesis would be normal: something similar seems to happen in the human foetus, in which the testicular intestitial cells are very large and rich in cytoplasm from the stage of 3 cm. to a stage of about 15 cm., after which they almost disappear (Gillman, 1948); masculine organogenesis was already complete.

The age factor in pituitary function

Research into hypophyseal function in the rabbit foetus was initiated in 1947, in order to verify whether the pituitary gland controls testicular activity. The foetuses were deprived of their hypophyses by the decapitation method, the value of which has already been discussed (Jost, 1951).

Briefly, it was noticed (Jost, 1951) that in the foetuses which were decapitated before the initiation of somatic sexual differentiation, testicular activity remained weak: on day twenty-eight the prostate was as reduced as in foetuses castrated on day twenty-one, and likewise the external genitalia were feminine.

The importance of the age factor in hypophyseal function was studied in two series of foetuses; some were decapitated early and killed at one-day intervals thereafter, others were decapitated at one-day intervals and killed at the same final stage (day twenty-eight). The analysis of the results indicates that the testis has some activity even in the absence of the pituitary gland. It works in a normal manner until approximately day twenty-two as judged from the first steps of differentiation of the genital tract; afterwards, mainly between day twenty-two to twenty-four, the pituitary gland is needed for normal testicular function; after day twenty-four it can be removed without suppressing masculine organogenesis.

Relationship between hypophysis and thyroid also indicated that, until the twenty-first or twenty-second day, the thyroid differentiation and specialization proceeds normally in the headless foetuses; afterwards the pituitary gland becomes necessary (Jost, 1953*a*, 1953*b*).

On the adult animal, the McManus technique (periodic acid - Schiff) has been shown to give indications about those pituitary cells which produce gonadotrophic or thyrotrophic hormone. Therefore this procedure was applied to the hypophysis of the rabbit foetus (Jost and Gonse, 1953). McManus-positive material accumulates in an increasing number of pituitary cells from day nineteen until day twenty-two to twenty-three and then diminishes in a significant manner; this last point will be illustrated here only by one case concerning litter mates, two of which were studied on day twenty-three and two others on day twenty-eight. The average numbers per section of distinctly stained cells, counted in three medial sagittal sections, distant 125μ, were respectively 232 and 263 on day twenty-three in comparison with 65 and 77

on day twenty-eight. It should be noted that the area of the sections almost doubles in the same time, which reduces the number of positive cells per unit of surface area in the oldest group. Such facts, verified on a rather large series of foetuses, strongly suggest that the activity of the pituitary gland passes through a maximum at a stage at which the testes and the thyroid request hypophyseal stimulation. Then the pituitary gland would not progressively increase its physiological work in parallel with the progress of its gross anatomical differentiation, but would release a larger amount of hormone during a limited period of time.

The incidence of the age factor on the relationship between hypophysis and adrenal cortex was studied with Miss A. Cohen on a small series of foetuses and some preliminary indications were obtained which require verification.

The rabbit adrenal cortex provides a less favourable material than that of the rat, its structure being less clearly defined and more variable. On day twenty-eight the cortex shows an external zone organized in large arcads and rather irregular internal cords, which centrally more or less penetrate into the medulla; other cortical cells are mixed with the medullary cells. In foetuses decapitated before day twenty-one or twenty-two and studied on day twenty-seven or twenty-eight the zone of arcads is relatively larger and the internal part markedly reduced. The ratio, width of the arcad zone to width of the internal part, is definitely in favour of the arcads, while in controls the internal part largely exceeds the zone of arcads. On the contrary, in foetuses decapitated on day twenty-six and studied on day twenty-nine, the internal part remained large. These preliminary observations seem to indicate that decapitating the foetus before the cortex is organized terminates in a more marked reduction than decapitating at later stages.

In the rat foetus the adrenal cortex grows markedly from day sixteen to day twenty-one; at about the age of nineteen days, modifications in the lipid content of the cortex suggest changes—probably an increase—in the physiological activity

of the gland (Cohen, 1954). Decapitation introduces a striking underdevelopment of the cortex (see Jost, 1954). A reduction in size of the cortex and some shrinkage of the cells occurs at birth (Josimovich, Ladman and Deane, 1954); alteration in the lipid content was observed by Miss Cohen (1955, unpublished data) in a few foetuses which had not been delivered at 22½ days of age. It might be wondered whether such changes are correlated with a reduction in the adrenocorticotrophic activity of the pituitary gland at the time of birth.

Glycogen storage in the liver

It is almost a hundred years since the discovery by Claude Bernard that the foetal liver stores appreciable amounts

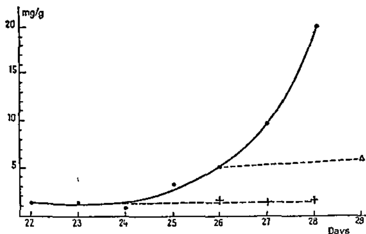


FIG. 1. Liver glycogen content expressed in mg./g. of fresh tissue; black points represent control foetuses; crosses represent foetuses decapitated between days 19 and 24 and killed on days 26 to 28; triangle represents foetuses decapitated on day 26 and killed on day 29.

of glycogen from a certain stage onwards when suddenly accumulation begins. In the rabbit foetus the turning-point appears on day twenty-five (Lochhead and Cramer, 1908, and Fig. 1). Although the question has not yet been

submitted to biochemical analysis, one may wonder whether at that stage the enzymic assortment of the liver cells undergoes a change and becomes adequate for storing glycogen.

Studies were undertaken to determine whether the change in the liver glycogen storage is hormonally controlled (see Jost, 1954). Measurements were made on decapitated rabbit and rat foetuses. Only the former will be considered here. It was first observed that in rabbit foetuses decapitated on day nineteen, the glycogen content was very low on day twenty-eight in comparison with litter mate controls (respectively $1 \cdot 58 \pm 0 \cdot 85$ mg./g. and $20 \cdot 06 \pm 3 \cdot 33$ mg./g. of fresh tissue; Jost and Hatey, 1949). The same result is also observed in all foetuses decapitated before or on day twenty-four and killed on days twenty-six to twenty-eight (Fig. 1; Jost and Jacquot, 1954, 1955).

In attempting to discover what hormonal factor was responsible for glycogen storage, different hormones were administered at the moment of decapitation. The detailed results will be presented elsewhere (Jost and Jacquot, 1955); briefly, it was observed that ACTH (Organon), in a dose of 2 to 8 mg. inserted under the skin, permits glycogen deposition in the decapitates, either at a normal or at a subnormal level, in fourteen out of sixteen treated foetuses. Two of these foetuses were thyroidectomized-decapitated, and they also gave positive results. An attempt was then made to obtain glycogen storage with corticoid hormones: no positive result was obtained with cortisone (3·5 mg.), hydrocortisone (3·7 or 4·1 mg.), desoxycorticosterone acetate (2 mg.), or 9-α-fluorohydrocortisone (25 μg.; high doses seem to be lethal), nor with the association of cortisone or hydrocortisone with DOCA or with 1 u. of insulin, or with 50 to 90 μg. of thyroxine nor with a small amount of growth hormone. Although only a small number of experiments have been carried out in each case, it is curious to notice that no other hormone than ACTH was found to permit glycogen deposition. If the adrenal glands are involved, one cannot discard the possibility that they produce some other com-

pound than those which were tried, or at other levels. In any case, the fact that giving ACTH can restore the physiological condition in decapitates allows the supposition that the glycogen storage in the liver is hormonally controlled.

It then would appear that endocrine factors introduce a turning-point in the carbohydrate metabolism, as they do in the properties of several tissues. In an effort to analyse the significance of this turning-point, it should be established to what extent the glycogen storage system has been changed by the first initial hormonal impulse. Attempts were made to study this point by decapitating after day twenty-five.

In four foetuses decapitated on day twenty-six and normally grown up to day twenty-nine, the mean value of the liver glycogen was 6·1 mg./g. (range: 3 to 8·7), while in controls the value was about 30 mg./g.; in eight 26-day-old control foetuses the mean was 5·1 mg./g. (range 2 to 7·85) (Fig. 1). So it [illegible]

store new glycogen at about the level to which it was brought before decapitation, since the values are expressed per g. of fresh tissue and since the liver continues to grow.

Further study is necessary to solve the question under discussion exactly. It should also be determined whether a liver, which has not been stimulated at the right time during foetal life, remains able to respond to delayed stimulation. Such problems involve implications for further postnatal life.

Conclusions

The aim of this informal paper was to draw attention to and perhaps to raise discussion on some suggestions resulting from experiments. Although many of the discussed problems are not yet solved, it seems that a pituitary-controlled hormonal impulse introduces a turning-point in the further properties of some tissues: the case of the sexual structures must be held as conclusive from this point of view. *Other tissues,*

such as the liver, also pass through a physiological turning-point, but the importance of this change for later postnatal life is not yet established.

The study of the pituitary gland in the rabbit or of the testicular interstitial cells in man, for instance, strongly suggests that such endocrine glands could pass through a period of maximal working at certain prenatal stages, followed by a period of lesser activity. It would then appear that the normal development of the foetus involves distinct endocrine events at appropriate stages.

Finally, although only one example of an actually transient tissue was considered in this paper, namely the Wolffian duct, the points which were discussed cannot be considered as too far from the main object of this colloquium, if the idea will be accepted that the term "foetus" does not define a particular state of an animal but covers a succession of different transient stages leading up to each other.

Acknowledgement.

We are indebted and wish to extend our thanks to several Laboratories which kindly supplied the homones used in these researches: Dr. Choay (Choay Laboratory) for growth hormone and insulin; Dr. Tausk (Organon, Holland) for ACTH; Dr. Velluz (Roussel—UCLAF), for cortisone, hydrocortisone, desoxycorticosterone acetate; Dr. Borman (Squibb) for 9-α-fluorohydrocortisone.

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DISCUSSION

[illegible]: It is evident from Professor Jost's account that the effects [illegible]

[illegible] decapitation?

[illegible] hydrocortisone given to the foetus was not too evident, since the placenta of the treated foetuses did not very clearly exceed the high range of individual variability. Have you any information on that matter, Prof. Huggett?

Huggett: With regard to controlling glycogen in rats and rabbits chemically, Prof. Jost is quite correct in saying that there is some factor causing considerable variation. But if you take a number of animals and

McManus-positive cells which he demonstrated in the pituitary. The material is very abundant at a certain time and disappears relatively rapidly at a critical period when you say testicular hormone is needed. Do you think there is a special release mechanism which is causing the discharge from the pituitary at that time, or do you think that the

that the same changes occur in the pituitary gland of the female and of the male foetus. The ovary does not seem to need gonadotrophic hormone. The pituitary gland could perhaps function in a self-differentiating manner.

Zuckerman: Prof. Jost, in elaborating your thesis about turning-points in the "determination" of tissues that are conditioned by pituitary hormones, it occurred to me that you might be able to alter their time relations by giving gonadotrophin before the critical days on which the pituitary hormones act (22 to 24).

of your hypothesis.

Villee: In any such system you have two parts—whatever is producing

respect it.

Zuckerman: Accepting all this, I am still wondering why in pursuing

the experimental analysis of the temporal phases of development, you [illegible]

[illegible]

still have to be done.

[illegible]

had a normal genital tract, another case which was doubtful, and two cases which were negative. So I should not like to give too much importance to these preliminary observations, but I feel that if it [illegible]

throughout reproductive life is very insecurely based. Since then, three sets of papers have appeared, stating the opposite view (see p. 30), and it is necessary to see how the work done in my own laboratory stands in the light of these newer studies.

The more important of our own observations, all consistent with the view that the female mammal usually begins her reproductive life with ovaries that are already furnished with a finite stock of oöcytes, are the following:

1. The total number of oöcytes declines sharply with age (rats: Mandl and Zuckerman, 1951*a*; monkeys: Green and Zuckerman, 1951, 1954).

On this point our observations merely confirm the careful studies of the rat carried out by Arai (1920*a*), as well as the impressions obtained by a number of other workers in somewhat sketchy studies of other species.

2. Oöcytes without any cellular cover, or surrounded by a single layer of cells, make up at least 90 per cent of those present in the monkey and rat (Green, Mandl and Zuckerman, 1951). Given that proper care is taken to enumerate all oöcytes, and that account is also taken of age and litter-relationships, no evidence can be found that the total number of oöcytes fluctuates with the phases of the oestrous or menstrual cycle (rat: Mandl and Zuckerman, 1950; monkey: Green and Zuckerman, 1951, 1954).

The contrary view, which has been taken to mean that there is a cyclical generation of oöcytes in the mature ovary, has been mainly based on impressions gained from the variations that occur during the course of the menstrual and oestrous cycle, not in the total population of oöcytes, but in the numbers of, and in the incidence of atresia in, large and medium-sized follicles. We have confirmed by numerical study that the latter changes do occur, but have also shown that no value can be attached to views about the neoformation of oöcytes in the adult ovary, or about cyclical variations in the intensity of oögenesis, that are based on counts from which the primordial germ cells are excluded. The same point is illustrated by

certain observations reported by Simpson and van Wagenen (1953), and in which purified FSH was administered to immature rhesus monkeys. Histological preparations of the ovaries gave the impression that this treatment stimulated oögenesis in all its phases, and that "small ovocytes" were formed in the germinal cords. Subsequent counts showed, however, that the total number of oöcytes present after treatment was much the same as in the ovaries of normal monkeys of comparable age. In a further set of experiments, in which FSH was administered to unilaterally-ovariectomized immature monkeys, it was found that the numbers of oöcytes in the hormone-stimulated ovary was of the same order as in the ovary that was removed before treatment began.

3. The number of oöcytes does not increase in successful autografts of ovarian tissue, reimplanted after freezing at very low temperatures (— 190° C) or after having been kept at normal room temperature (Green, Smith and Zuckerman, 1956).

To the best of my knowledge, this study is the only one so far carried out in which the numbers of oöcytes were counted in ovarian grafts. The conclusion to which it points is in line with what is said below about fragments of ovarian tissue left in the body *in situ*.

4. Oöcytes can survive for 2½ months in ovarian homografts in rats, and for a year or more in autografts in monkeys, in the absence of the germinal epithelium (Breward and Zuckerman, 1949; Mandl and Zuckerman, 1949).

Here, our observations merely confirm a number of earlier ones made by other workers (e.g. Herlitzka, 1900; Marshall and Jolly, 1907; Pettinari, 1928). The indication is that oöcytes may have a very long life.

5. The total number of oöcytes in the one ovary of rats from which all traces of germinal epithelium had been removed by means of the application of corrosive fluids was not significantly different, over a period of nearly 1½ years, from that in the untreated normal ovaries of the same animals. Correspondingly,

the total number of follicles in both ovaries of the treated rats was only slightly lower than the expected number in normal rats of the same age (Mandl and Zuckerman, 1951*b*).

The results of this experiment extend a corresponding study reported by Moore and Wang (1947), and the conclusion to which both point is that the cellular division which may be observed in the germinal epithelium of adult ovaries bears no necessary relation to the process of oögenesis.

6. X-irradiation of rats and mice leads to the disappearance of all oöcytes from an ovary, without at the same time causing any definable histological or cytological change in the germinal epithelium (Humphreys and Zuckerman, 1954; Mandl and Zuckerman, 1956*a*, *b*).

These findings confirm many earlier observations (e.g. Lacassagne, 1913; Brambell and Parkes, 1927; Everett, 1943); Everett believes that the germinal epithelium of the mature mouse, the animal on which he experimented, consists of cells which are somatic in origin, as well as primordial germ cells that originate in the gut entoderm, and which are set aside during early embryonic development. Without providing any cytological evidence to support his view, he suggests that X-irradiation destroys the germinal epithelium without affecting the somatic elements.

7. Compensatory hypertrophy of an ovary is not associated with an increase in the total number of oöcytes, but the single hypertrophied ovary contains as many follicles with antra as do the two ovaries of a normal animal (Mandl and Zuckerman, 1951*c*).

These findings confirm Arai's (1920*b*) earlier study of compensatory hypertrophy in the rat. They also accord with Lipschütz's observations (1925, 1928; also Lipschütz and Voss, 1925) on the cat and rabbit. These indicated that no new oöcytes are formed when an ovary or fragment of an ovary undergoes compensatory hypertrophy, and that the number of oöcytes present in a small piece of ovarian tissue left in the body is gradually and rapidly exhausted by recurrent phases of follicular maturation.

8. The conclusions stated in the preceding paragraph have been confirmed (Mandl, Zuckerman and Patterson, 1952) in a more extensive series of experiments on litter-mate rats which received the following treatments:

A. right ovary removed: more than half of left resected.
B. right ovary removed: left ovary untreated.
C. same as A, but remaining fragment of left ovary painted with tannic acid in order to destroy the germinal epithelium.
D. right ovary removed: surface of whole left ovary painted with tannic acid.

Compensatory hypertrophy occurred in the ovarian tissue left in the body, whether or not the germinal epithelium was present, and was not associated with any increase in the expected number of oöcytes. At the same time a unilaterally-ovariectomized animal lost fewer oöcytes within a given period than a normal animal possessing both ovaries. But the rate of loss was substantially greater than would be expected to occur in a single ovary of a normal animal, and increased inversely with the amount of ovarian tissue left in the body.

The latter observation suggests that the smaller an ovarian graft, the more quickly will it become depleted of its oöcytes.

9. The total number of oöcytes does not increase after hypophysectomy (Ingram, 1953).

This experiment was carried out in order to test statements by Swezy (1933) that stimulation by gonadotrophic hormone decreases the rate of oögenesis in the adult rat, and correspondingly, that the rate of oögenesis increases after hypophysectomy. The hypophysectomized animals which she used were, in fact, younger than her control animals, and would have been expected to possess more oöcytes, regardless of other physiological considerations. Her data merely suggest that the number of oöcytes decreases more slowly with age in hypophysectomized rats than in normal animals.

These are the main observations bearing on the problem of

oögenesis which have so far emerged from studies in my own laboratory. It is necessary to consider them in the light of the contrary statements that have been made more recently by Aron, Marescaux and Petrovic (1952, 1954*a*, *b*); by Van-Eck (1955); by Burkl (1954, 1955); and by Burkl and Kellner (1954*a*, *b*, 1955).

The new observations reported by Aron, Marescaux and Petrovic (1954*b*) relate to the apparent occurrence of differentiating oöcytes in the ovary of the mature guinea pig. They present their findings in the context of a general review, in which they refer to various statements made by other workers in support of the alternative views (a) that oögenesis occurs only during embryonic life; (b) that it continues for a variable period after birth; (c) that all the oöcytes formed during embryonic life are destroyed shortly after birth, and are then replaced by a new generation of oöcytes; and (d) that new oöcytes are formed throughout reproductive life. Aron and his collaborators come down firmly in favour of the last of these hypotheses, and cite a number of papers which they believe prove that oöcytes can be formed either from the germinal epithelium or from cord-like invaginations of this epithelium within the substance of the mature ovary. Their selection of the literature is, however, arbitrary; and they make little attempt to analyse critically the papers they cite. Indeed, at least a few are quoted in a sense different from what their authors apparently intended. For example, Nunes (1932) is referred to as one who has helped establish " with certainty" that oöcytes differentiate from invaginations of the germinal epithelium within the substance of the ovary. In fact, all his paper records is that in the adult rabbit ovary follicles are often closely related to epithelial invaginations, and his own conclusion—"on ne peut donc nier d'une manière absolue la néoformation ovigène dans l'ovaire adulte"—is much less categorical than is implied by Aron *et al.*'s references. Lane-Claypon (1905) and Pincus and Enzmann (1937), who are among many others who have studied the rabbit ovary, and both of whom are also cited by Aron *et al.*, reached quite

different views about oögenesis—Lane-Claypon that oöcytes were formed from interstitial cells, and Pincus and Enzmann that not only was this not the case, but that few mitoses occurred in the germinal epithelium, and that no primordial oöcytes migrated from this epithelium. In themselves, these differences of view merely indicate that the so-called histological evidence of oögenesis is anything but clear-cut. But the fact that they exist makes it reasonable to expect some justification for giving greater weight to one rather than another interpretation of the histological data. This Aron *et al.* do not do.

Matthews' and Harrison's (1949) observations on the seal are also cited as cast-iron evidence that invaginations of the mature germinal epithelium give rise to oöcytes. But on this point, these two authors are completely non-committal. All they say is that "in many instances the smaller crypts, and diverticula of the larger ones, are found to terminate as primary follicles, the epithelium surrounding the oögonia being directly continuous with the germinal epithelium lining the lumina of the crypts. . . . It is not yet clear whether the oögonia arise directly from the epithelium of the crypts or whether they have reached their position previously from some other source."

Other authors, again, are quoted as stating that oöcytes may be present between the cells of the germinal epithelium of the mature ovary, the implication being that they are formed there. The observation is one which must be familiar to all who have studied the ovary. It is, however, another and purely arbitrary matter to infer that the presence of oöcytes in this situation indicates that they are derived from germinal epithelium. Furthermore, it is not at all clear that many of the authors cited by Aron *et al.* themselves regarded, or would now be prepared to regard, their observations as indicative of this particular conclusion. For example, all that Deane (1952), one of the workers referred to, states is that "small ova are occasionally seen within the germinal epithelium—more frequently they occur just *below it, accompanied*

by a cluster of satellite cells." The further inference about oögenesis, implied by Aron *et al.*'s reference to the paper, is not even mentioned. The only paper cited in connection with this particular issue, and which, in fact, bears critically on the question of oögenesis, is that of Hamlett (1935) on the armadillo. It does so, not because it emphasizes the topographical relations of oöcytes to the germinal epithelium, but because it describes nuclear changes that may be indicative of oögenesis (see later).

Observations about the position of cells in the ovary are far too equivocal to sustain conclusions about the occurrence of oögenesis in adult life. Oögenesis is a process. As I have emphasized before (Zuckerman, 1951), to infer that it occurs from a study of histological sections implies that separate and distinct phases of the process, each necessarily viewed in isolation can, as it were, be set in continuous motion by a dynamic interpretation. This might be a reasonable exercise if there were clear-cut and generally accepted cellular stages which in the mature ovary linked an indisputable oöcyte with some other and equally well-defined cellular constituent of the ovary, for instance, a cell of the germinal epithelium. But this is not so. Some writers, for example Allen (1923) and Bullough (1942), have merely assumed that if a cell of the germinal epithelium divides, one or both of the daughter cells will differentiate into an oöcyte, and only a very few have attempted to define the cellular stages which, in the adult ovary, might link an oöcyte with a cell of the germinal epithelium, or with any other cellular component of the ovary. The majority of histologists have completely failed to convince themselves on this point.

I have elsewhere suggested an alternative, and far simpler, explanation of the proliferative powers of the germinal epithelium (Zuckerman, 1951). It is that the capacity of the cells of the adult germinal epithelium to subdivide reflects the extensive cyclical changes which occur in the shape and size of the ovary as follicles mature and burst, and tear the surface of the ovary, and as corpora lutea develop. These

changes could not possibly run their course in the way they do if the germinal epithelium was not able to proliferate (see also Schmidt and Hoffman, 1941; and Schmidt, 1942).

Other kinds of evidence bearing on the question of oögenesis are referred to by Aron *et al.* in a non-critical, or at best non-committal way, and apart from certain comments which are referred to below, these authors do not resolve the contradictions posed by the work done in my own laboratory to the thesis they support. They proceed to deal with the field of observation in which they themselves have contributed. This concerns the occurrence in the ovaries of the mature lemur, the armadillo and the guinea pig, of nuclear changes which are suggestive of the division of oöcytes. The lemurs can be usefully considered first, as they were the first to be described in this connection (Gerard, 1919/20; Rao, 1927; and Gerard and Herlant, 1953).

The observations of Gerard (1919/20) and Gerard and Herlant (1953) relate to adult specimens of the African Lorisidae (*Galago senegalensis moholi, G. crassicaudatus, G. demidoffi* and *Perodicticus potto*). In all these species "oögenetic cords" containing primordial oöcytes are found separated from the germinal epithelium by a tunica albuginea of varying thickness. Many of the cells in these cords are actively dividing, and according to Gerard and Herlant can be regarded only as oögonia. Other cells show chromatin configurations characteristic of the leptotene, synaptene and pachytene stages of meiosis. Cells corresponding in all cytological respects with oögonia were also recognized in the germinal epithelium itself.

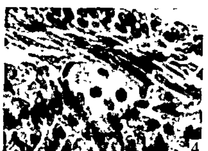
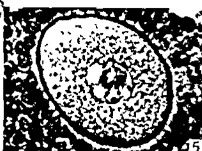
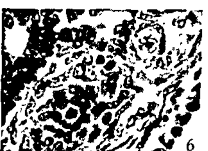
Rao (1927) studied the Indian species *Loris lydekkerianus*, and noted that "tubular" invaginations of the germinal epithelium ramify in certain parts of the ovary of the pregnant, but not non-pregnant, adult. Rao was under the impression that it had been firmly established that oögenesis in mammals continues throughout adult life, and he therefore tried to decide whether oöcytes are formed from invaginations of the germinal epithelium, or as Lane-Claypon had suggested in the case of the rabbit, from the interstitial cells of the ovary as

well. His own view was that cells from the germinal epithelium divide mitotically, and either give rise to granulosa cells or pass into the interior of the ovary as interstitial cells, where they may later become transformed into oöcytes. He describes how the nucleus of an interstitial cell may enlarge, with the chromatin filaments of the nucleus separating over the increased area in the form of a loose meshwork interspersed with chromatin nodules. This he regarded as the leptotene stage of meiosis. He then goes on to describe what he considered to be a synaptic condition of the nucleus, indicated by the massing of the filaments into a lump on one side of the nucleus. A pachytene stage is also pictured, in which the synaptic lump is resolved into coarser and more bulky filaments. On the other hand, Rao states that the diplotene phase is either fugitive or does not occur, since the dual arrangement of the filaments cannot be made out. All these interpretations are based entirely upon an analysis of separate cells, and Rao is careful to point out that he did not have the opportunity of studying oögenesis, for purposes of comparison, in foetal ovaries.

Hamlett's (1935) observations of oögenesis in the mature armadillo relate to a single pregnant specimen which had "a button of tissue projecting abnormally from the otherwise smooth surface" of the ovary. His histological description accords closely with that of Gerard and Herlant for the lemur, but Hamlett, nevertheless, regards his specimen as illustrating not the normal state of affairs, but "what must happen in the rare instances" when the epithelium of a mature ovary in which the tunica albuginea is well developed "undergoes proliferation". "The cells are forced outward, not inward, for they are incapable of penetrating the dense layer of connective tissue and smooth muscle forming the tunica." He goes on to say that "any postnatal replenishment of oöcytes" from the surface is normally impossible in the armadillo, as well as in other animals whose ovaries are invested by a dense tunica.

The oögenetic phenomena described by Gerard and Herlant and by Hamlett seem, at first reading, to be similar to those



Oöcytes in Immature and Mature Guinea Pigs

Fig. 1. Immature guinea pig. Nest of oöcytes showing phases of meiosis, probably pachytene and diplotene.

Fig. 2. Pubertal guinea pig. Nest of oöcytes showing phases of meiosis, including zygotene.

Fig. 3. Mature guinea pig. Nest of oöcytes showing phases of meiosis, including apparent zygotens.

Fig. 4. Old guinea pig. Nest of oöcytes showing phases of meiosis, including apparent zygotene.

Fig. 5. Normal oöcyte, showing pachytene phase of meiosis.

Fig. 6. Three stages of meiosis.

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which Aron and his collaborators now report in the guinea pig, and which we can confirm from personal observation. One often finds in the ovarian cortex of this animal, cells, frequently crowded together in nests, which seem to be either oögonia or oöcytes. What appear to be phases of meiosis can

mitosis, their view being that oögonia, having been formed by a straightforward metamorphosis of quiescent germinal elements in the germinal epithelium, become directly transformed into oöcytes. Aron *et al.* also believe (a) that this presumed neoformation of oöcytes varies considerably from animal to animal; (b) that until the animals are about 2½ years old, when it usually becomes negligible or ceases, the process is independent of age; (c) that the process does not vary *cyclically; (d) that its intensity is not affected either* by gonadotrophin or hypophysectomy; (e) that it does not occur more vigorously in one ovary after its fellow has been removed; and (f) that it is not affected by the injection of oestrogen.

What is significant about these observations is not that *Aron et al. were able to observe phases of meiosis* in nests of oöcytes, but the fact that they failed to find any evidence of mitosis of oögonia, and that they were consequently forced to conclude that the latter are formed from the direct transformation of dormant cells in the germinal epithelium. Others *before them,* as well as ourselves, have also failed to observe

(e.g. nests of "oöcytes") seen in the guinea pig ovary have lent themselves to a variety of interpretations, and that one group of workers, following Loeb (1905, 1932) regard parthenogenetic development of ovarian oöcytes as common in this

course, conceivable that the nuclear changes described by Aron *et al.* in the ovary of the mature guinea pig constitute no more than the meiotic changes which usually occur prepubertally in most other mammals. For example, de Winiwarter (1920) has noted that the reduction division of oöcytes may occur in the ovaries of cats which have just passed puberty. To the best of my knowledge, however, active meiotic changes are not usually seen, except in degenerating follicles or follicles about to ovulate, in the ovaries of adult rats, mice, rabbits, ferrets and monkeys.

None of these reports, indicating or suggesting that meiosis, and in Gerard and Herlant's case mitosis as well, may occur in the germinal cells of the ovaries of mature females of certain species, is furnished with a control account of the histology of the foetal ovary, at those times when oögonia are unquestionably multiplying. This deficiency was recognized by Rao, but is not referred to by the other workers concerned. Bujard (1947) describes the ovary of the immature guinea pig, but nothing he records allows one to determine the extent to which the presumed oögenetic phenomena in the adult ovary, *as outlined by Aron et al., and as confirmed by ourselves,* correspond with the histological manifestations of actual oögenesis, which presumably is still in progress at birth. Oögenesis in the mature animal must by definition mean the continued formation of several oöcytes from some precursor cell, in the same sense that a single spermatogonium is in the end responsible for the production of a very large number of spermatozoa. An essential criterion of oögenesis would, therefore, have to be the multiplication of oögonia by mitosis, in the same sense that the occurrence of meiosis represents the end, not an intermediate phase, in the process of formation of the ovum.

The studies reported by Burkl (1954, 1955) and Burkl and Kellner (1954*a*, *b*, 1955) are in general of a more experimental kind than those of Aron *et al.* In his first paper, Burkl (1954) reports that oestrogen (hexoestrol implantation) increases the rate of neoformation of oöcytes in rats, as judged by the

"activity" of the germinal epithelium. This conclusion is hardly borne out by the data that are published, since these, in fact, show that the total number of primordial oöcytes was smaller in treated animals than in controls of the same weight-groups. Results of experiments in which rats were fed the highly toxic drug Myleran are also taken by Burkl and Kellner (1954*a*, *b*) to support the thesis that oögenesis is a process which continues into adult life. Some of the animals used in this experiment died as a result of the treatment, and only one normal and three experimental rats were left to provide what in effect proved to be the basic thesis of the study, that Myleran acts only on follicles whose oöcytes exceed 38μ in diameter. This figure is used in a series of calculations designed to determine the daily maturation rate of follicles, by which is meant the number of oöcytes over 38μ in diameter divided by the period of the experiment. By successive and increasingly unconvincing transformations and comparisons, the conclusion is then reached that some 25 oöcytes degenerate in each cycle. Since the stock of oöcytes at the onset of sexual maturity is asumed to be 3500, it is concluded that neoformation of oöcytes must occur if the ovary is not to be depleted of oöcytes too soon. Quite apart from various theoretical and mathematical defects of the argument, the design of the whole experiment is totally inadequate to sustain any such conclusion. It is worth noting, too, that the average number of oöcytes at puberty in the Birmingham strain of albino rats is 6000, and that a stock of oöcytes of this size would be sufficient to sustain the rate of loss of oöcytes calculated by Burkl and co-workers without assuming that oögenesis continues after sexual maturity had been reached.

In a later paper, Burkl (1955) again concludes, but on this occasion without resorting to oöcyte counts or a study of nuclear changes, that oögenesis occurs in the mature ovary of the dog. This he does merely on the basis of the topographical relation of oöcytes to cords of epithelial cells which had presumably been derived from the germinal epithelium. And in a paper which follows (Burkl and Kellner, 1955), he

purports to show that the rate of neoformation of oöcytes in albino rats increases after hypophysectomy. Oöcyte counts are given for this study, but their statistical analysis, again, does not bear out the conclusion stated. In the first experiment the mean number of oöcytes for eight prepubertal hypophysectomized animals was 3840 ± 300, and for five controls 3180 ± 170. This difference is not significant statistically. In a second experiment, carried out on mature animals, the corresponding figures for eight hypophysectomized animals are 2650 ± 230 and for five normal controls 2580 ± 230. Again, the difference does not approach statistical significance. Analysis of these figures, in fact, shows that the results of Burkl's experiments agree closely with those of Ingram (1953), carried out in my own laboratory. They fail to demonstrate a significant increase in the number of oöcytes after hypophysectomy, but do indicate that the usual rate of decline in the numbers of oöcytes may slow down after the pituitary is removed.

Van-Eck (1955) attempts to reach a conclusion about the occurrence of oögenesis in the adult ovary of the rhesus monkey from an experimental estimate of the time it takes an oöcyte to become atretic and to disappear. This work is based on oöcyte counts of fourteen immature (representing ten animals) and seven mature ovaries (representing four animals). The time taken for a follicle to become atretic was estimated from experiments on three young adult monkeys which were irradiated with 1200 R (600 R on two successive days) between the 19th and 22nd day of the menstrual cycle, and ovariectomized 7, 10 and 14 days later respectively. Oöcytes surrounded by more than one layer of cuboidal cells were not present after 7 days, and after 14 days the only oöcytes present were those surrounded by one layer of epithelial cells. Van-Eck concludes that "once atresia occurs in a follicle the process is invariably completed within two weeks; for the small growing follicles the process takes less than one week."

On the assumption that the time taken for atresia to occur

is the same "whether the damage is caused physiologically (from unknown factors) or artificially (by X-rays)", and on the basis of estimates of the total number of normal oöcytes, and the percentage of oöcytes regarded as atretic, the following function

$$t_n = a(1 - r)^n$$

in which t_n = the number of oöcytes after n periods of atresia; a = the original number of oöcytes; and r the percentage of atresia, is used to show that the ovaries would be practically exhausted within two years if new oöcytes were not formed; consequently, oögenesis must occur in the mature ovary, two years being the theoretical maximum life of the oöcyte.

Once again both facts and arguments are hardly adequate to support the conclusion. In the first instance, Van-Eck assumes that normally "atresia is of short duration for the primordial and small primary follicles—the follicle is resorbed, leaving no trace, and its place in the ovary replaced with stroma." To the best of my knowledge this contention is arbitrary, in so far as no critical proof has ever been provided that under physiological conditions atresia of young oöcytes is a rapid process. Estimates of the rate of atresia, inferred from observations on single ovarian phases, are just as insecure as are inferences of postpubertal oögenesis from a picture of an epithelial cord apparently budded from the germinal epithelium. No-one knows how long an atretic oöcyte or follicle in a normal ovary can remain in the same apparent state; nor indeed is there any agreement among students about the occurrence of a high rate of degeneration in primordial oöcytes.

A second weakness of the work is that it assumes that atretic oöcytes can be diagnosed sufficiently well to justify statements such as: "the percentage of atretic ova was constant for each group". (According to her estimates 4·5 per cent of all oöcytes are atretic or in process of becoming so.) Elsewhere in the paper, however, she noted that "for the primordial oöcytes it is sometimes difficult to determine

whether the cell is atretic or still normal"—and in such cases she counted the cell as normal. Actually, anyone who has tried to make successive counts of the atretic oöcytes in an ovary will know how numerous the marginal cases are, and how difficult it is to obtain estimates which agree closely.

A third criticism of Van-Eck's study is the unwarranted assumption that the atresia which occurs as a result of X-ray sterilization is identical in its nature, and in its duration, with what occurs in the normal ovary. The only specific enquiry into this point of which I know (Halberstaedter and Ickowicz, 1947), in fact, indicates that the first histological appearances of atresia in the normal ovary are characteristically different from those which herald atresia after X-irradiation. An additional difficulty is that Van-Eck implicitly assumes that the time taken for an oöcyte to disappear after X-irradiation is independent of the R dosage. The three monkeys which she irradiated were given a total of 1200 R in two applications through the dorsolumbar region of the body wall. It would be extraordinary if the rate at which oöcytes disappeared were not increased if the same dosage were applied directly to the ovaries, or alternatively, if a dose five times as great were applied directly (see Lacassagne, 1913).

The question whether oögenesis does or does not occur in the mature animal has been so beset with equivocal fact and arbitrary interpretation, that it is essential that new observations on the topic, which clearly has important practical implications, should be marked by observational and conceptual precision if they are not to multiply confusion. The first point that needs to be settled is whether the experimental and biometric data summarized on pp. 32–35 are consistent with any thesis other than that oögenesis is in abeyance in the mature rat or monkey. By "settling", I do not, of course, mean the matching of fact with speculation—for example, disposing, as do Aron *et al.* of the observation that oöcytes can persist in an ovary long after it has lost all recognizable signs of its germinal epithelium, by suggesting that it is possible that some unrecognizable or undefined derivative of that epithelium

is still persisting within the substance of the ovary. What we need to know is whether any thesis other than that a finite stock of oöcytes is involved can be reconciled with such plain numerical propositions as "the total number of oöcytes does not increase in an ovary undergoing compensatory hypertrophy"; or "the relative rate of loss of oöcytes increases inversely with the amount of ovarian tissue left in the body". To some extent Aron *et al.* appreciate this point, and their way of dealing with it is to suggest, in effect, that the smaller the amount of ovarian tissue left in the body, the more the work it has to do in satisfying the body's need for oöcytes, and consequently, the more rapid the decline in the oögenetic potency of its germinal epithelium. The difficulty with this hypothesis is not that it is arbitrary; it is unverifiable. The only tangible measure of oögenetic potency which one can conceive of would be the numbers of oöcytes produced in the ovary at any given time. As I have tried to show, any realistic analysis of the differences which occur in the numbers of oöcytes in different experimental conditions, or as between different normal physiological states, leads inevitably to a conclusion which implies a primary stock of oöcytes. The other possibility is that oöcytes are transitory structures, and that the actual ones that are seen, and which could be counted under the microscope at any given moment, would have been replaced by another set of oöcytes had the ovary been examined on a later occasion (e.g. that the number counted on one occasion was the measure of a given oögenetic potency at that moment; and a lesser number counted in another set of comparable ovaries on a physiologically later occasion was a measure of a lesser oögenetic potency). This, in fact, is what *is implied by Aron's suggestion. If it were accepted, no more* would be demanded of one's credulity than would be from a man who counted the apples on a tree on successive days, and who held that each day he was counting a set of apples which had miraculously replaced those that were there the day before—and just as miraculously disappeared (there being no fallen apples to account for the new ones that had

suddenly appeared). To some extent it is a matter of taste which view one accepts; my own is for the simpler proposition that the identities of apples—and equally of oöcytes—cannot be so simply switched.

What answer should, then, be given to the question whether oöcytes continue to be formed in the adult ovary? If we set aside for a moment some of the cytological evidence, the experimental data undoubtedly support the view that as a rule the mature mammalian ovary is incapable of adding to the store of oöcytes with which it is furnished by the time of puberty. Most of this evidence has been derived from work on rats, but the more fragmentary observations that have been made on other species agree far more with this thesis than they do with the contrary one. There appear to be certain notable exceptions, and it may well be that oögenesis ceases in some species earlier than in others, in which it may continue for some time after puberty. But in general the ovary appears to be a transient tissue which, so far as its main function of oögenesis is concerned, has little or no powers of regeneration once puberty has been reached. Compared with the testis, the ovary is in this respect a structure which reaches *senescence early, and which cannot be rejuvenated by any* known hormonal treatment.

Ovarian secretion

This limitation does not extend, in any corresponding measure, to the ovary's powers of hormonal secretion. These normally manifest themselves first at the time of puberty, and in woman they fade out at the menopause. In the interval between these two temporal events, the ovary's capacity to secrete hormone is under the control of the gonadotrophic secretions of the pars distalis of the pituitary. Its capacity to respond to this stimulation is often held to be independent of the presence of germinal elements, but the evidence bearing on this point is somewhat equivocal. Thus, in rats, an ovarian graft that is apparently devoid of oöcytes may sometimes secrete sufficient oestrogen to provoke continuous or

recurrent oestrous phases in the host animal for periods up to a year (Parkes, 1950). Similarly, mice which have been sterilized by X-irradiation, and whose ovaries are devoid of oöcytes, may still continue to experience recurrent phases of oestrus for periods up to 69 days (Parkes, 1927). On the other hand, it is a matter of common observation that the postmenopausal human ovary, when depleted of its oöcytes, ceases to secrete sex hormone. And it is also believed that it cannot do so, even when subjected to the influence of gonadotrophin (see Burrows, 1949). Because of the apparent conflict between these two sets of observations, the whole subject has recently been re-investigated in my own laboratory.

The first point which we (Mandl and Zuckerman, 1956*a*, *b*) re-investigated was the observation that a mouse ovary that has been depleted by means of X-irradiation of all oöcytes and of all recognizable follicular elements may still produce sufficient oestrogen to induce vaginal cornification and even recurrent oestrous cycles (Parkes, 1926, 1927; Westman, 1930). Our technique differed from the earlier experiments in so far as we irradiated the ovaries not through the body wall, but directly. Only ovaries which were found on serial section to be absolutely devoid of oöcytes were regarded as sterile. According to our observations on rats, the usual pattern of oestrogenic activity after successful irradiation (as judged by the criterion of a total lack of oöcytes) is that short periods of vaginal cornification occur at increasingly irregular intervals, and that after a minimum of five and usually within 40 days, the vaginal smear becomes continuously cornified. The period of cornification lasts from 2 to 14 weeks, and during this time the animals, in our experience, will not mate. The vaginal smear then becomes anoestrous in type. The behaviour of the X-ray sterilized mouse also follows this pattern. Signs of oestrogenic stimulation are not confined to the vaginal epithelium, but can also be observed in the uterus, which while smaller than normal, remains significantly larger than in spayed control rats as long as 26 weeks after treatment. On the other hand, changes occur in the weight of

the body, thymus and spleen during the three months which follow sterilization, which correspond to those that occur after surgical removal of the ovaries. Further experiments showed that these indications of oestrogenic activity continue after bilateral adrenalectomy, but cease after removal of the X-ray sterilized ovaries—which presumably are therefore responsible for the secretion of the hormone.

In effect, what all this adds up to is that the capacity of the ovary to secrete sex hormone is neither necessarily, nor absolutely, dependent upon the presence of germinal elements. Earlier histological studies of the ovaries of mice that had been X-irradiated at birth or at the age of three weeks had indicated that the cells which are responsible for the continued secretion of oestrogen after the elimination of the follicular system are those which are derived from what is described as "the first post-irradiation proliferation" of the germinal epithelium (Brambell, Parkes and Fielding, 1927*a*, *b*). The tissue produced by this proliferation is "almost indistinguishable from true luteal tissue . . . the epithelial cells becoming like luteal cells with connective tissue elements of the sheath growing in amongst them like the thecal cells of the corpus luteum." Such proliferations do not occur in the adult ovary. The X-irradiated adult mouse which continues to undergo oestrous cycles was held by Brambell and Parkes (1927) to be under the influence of oestrogen produced by "interfollicular tissue" and "follicular derivatives". The latter two elements are, for all practical purposes, indistinguishable from each other, and form the main part of the sterilized ovary. Histologically they closely resemble the cells seen in mice irradiated before puberty, and which are derived from the cords of the first proliferation.

We have found that even when no luteal-like cells are present in rats sterilized before puberty, and when no cyclical changes occur in the vaginal smear, the vaginal closure membrane nevertheless breaks down (Mandl and Zuckerman, 1956*c*). Since treatment with oestrogen makes it do so precociously (Allen and Doisy, 1924), the obvious inference would be that

the sterilized ovary secretes a small amount of oestrogen even when no obvious luteal cells are present, and that the threshold of oestrogenic stimulation responsible for canalization of the vagina is lower than that at which cyclical changes in the vaginal epithelium occur. An alternative possibility is that an X-irradiated ovary which is devoid of apparent luteal tissue does not produce oestrogen but androgen, or some adreno-cortical hormone, and that the latter is responsible for the break-down of the vaginal closure membrane (see Burrows, 1949). Whichever hormone it be, there is little likelihood that it merely accelerates the canalization of the vagina, which would otherwise take place, independently of hormonal action, as development proceeds, since, according to our experience, the vaginal canal remains permanently closed if rats are ovariectomized twenty days or so before the usual time of disappearance of the closure membrane.

One further fact needs to be considered before returning to the question of the dependence of the secretory function of the ovary on its germinal elements. Is the secretory capacity of the ovary after X-irradiation, whether slight or pronounced, under the control of pituitary gonadotrophin, or is it autonomous? The answer is not clear-cut, but inclines to the latter possibility. We have found, in experiments on rabbits and mice, that an ovary that has been effectively sterilized by means of X-rays, and in which all oöcytes and follicles have been destroyed, does not undergo compensatory hypertrophy when its fellow is removed (Humphreys and Zuckerman, 1954). This observation has been confirmed in a more extensive series of experiments on rats (Mandl and Zuckerman, 1956*a*). These experiments have also shown that a completely sterilized ovary not only fails to undergo compensatory hypertrophy, but also does not respond by an increase in size to exogenous gonadotrophin. On the other hand, we also have indications—they are little more than this—that the intensity of oestrogenic secretion of the X-irradiated ovary, however slight it may be, can be increased by gonadotrophic stimulation.

These observations seem to leave little doubt that the mechanism of secretion is different in an X-irradiated as compared with a normal ovary. Not only does it not respond to gonadotrophic stimulation like a normal ovary, but at no time (to judge, for example, by the size of the uterus) does it secrete as much oestrogen as a normal ovary. Since neither recurrent periods of vaginal cornification nor persistent cornification continues indefinitely after X-irradiation, it is also clear that the X-irradiated ovary becomes progressively incapable of secreting at all. This conclusion leaves open the general question whether the decline in secretory capacity is due to the elimination of the germinal elements of the ovary, or to damage inflicted on other ovarian cells by the X-irradiation. At the moment it is difficult to see how this particular problem can be answered. A decision could possibly be based on experiments in which rats are irradiated in a carefully graded series, so that their post-irradiation behaviour could be related to the amount of damage suffered by their ovaries.

Conclusion

From the fact that reproductive life in higher mammals is of shorter duration than life itself; from the knowledge that fertility in mammals, including man, reaches a peak in early maturity and then gradually declines; from the observation that the senescent ovary cannot be reactivated by any known hormonal treatment; and from the experimental data reported in this paper, one can only conclude that in general the ovary is a transient tissue with little regenerative capacity, not only with respect to its germinal but also to its secretory functions. The period over which the ovary is a regenerating structure, in the sense that it is able to produce oöcytes, may vary from one mammalian species to the other, but, in general, it is an organ which is more senescent, or potentially more senescent, than the testis, both from the germinal and the secretory points of view.

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DISCUSSION

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see any convincing production of oöcytes in mature monkey ovaries. This is in spite of Swezy's account based in some part on some misinterpreted specimens from one of my papers. I do not think she ever saw the specimens; she worked from my figures only.

In the recent work of Van-Eck done in part on some of the materials borrowed from me, there seems to me a possibility of a fundamental error. She tried to wipe the slate off, so to speak, by irradiating the ovaries and then used deductions about the growth of follicles immediately thereafter, without apparently realising the possibility that the irradiation had done something to those follicles which grow and degenerate in the next few days or weeks. So there again I find myself fully in doubt as to the neogenesis of ova.

Zuckerman: I think the flaw in the Van-Eck papers is really a serious one. Van-Eck has assumed, in the first instance, that the atretic phenomenon which occurs after irradiation is identical with natural atresia. Because you can destroy an oöcyte quickly with X-rays, she therefore assumes that you can tell exactly how long it takes for an ordinary ... There is no

... connection, ... al and pre-pubertal seem to be fundamental. What constitutes puberty from the point of view of regeneration of oöcytes?

Zuckerman: In the context of oögenesis the term varies according to species. If, for example, it is true that the lemurs can produce oöcytes when sexually mature, I would be inclined to say that they constitute one extreme in a range of mammals, the other being those species which achieve their entire complement of oöcytes before birth. In the case of the rat we know that nuclear configurations of the kind which could be interpreted as oögenesis cease to appear after about day 4. Some people put it even earlier.

Parkes: That is long before the first ovulation?

Zuckerman: Yes, but even so it would not surprise me to find a few oöcytes being formed later.

Huggett: Tell me, have you any knowledge as to where these oöcytes come from?

Zuckerman: I have always assumed that it was true that the gonocytes migrate into the embryo. Bounoure, Regan, Wolff and Nieuwkoop

Corner: Hurdig has been able to mark, in human embryos, the gonocytes with one of the microchemical stains—a lipophosphatase. This seems to be quite clear. Do you not think it is quite believable work and that this corroborates the old story?

Strauss: Is it possible for the neoformation of oöcytes in the adult mammal to be a question of comparative embryology as well as one of a cytological nature?

Zuckerman: In what sense embryological?

Strauss: In the lower archaic mammals, like the Galago and Dasypus, you will find some neoformation. Gérard, as well as Hamlett, claims to have seen neoformation in these primitive mammals, in as distinct a manner as one could wish.

Zuckerman: All these creatures started off on their reproductive life

a primitive phenomenon.

Matthews: In fishes, of course, there is entirely new production of oöcytes, but I don't know what happens in the bird.

Parkes: Prof. Zuckerman, can you say whether or not endocrine activity on the part of the ovary continues after the destruction of the oöcytes and, therefore, of the follicular system, and if so for how long?

Zuckerman: I can only give you the facts for experiments on rats

recognizable oöcyte in serial sections. Most animals entered a phase of

[illegible] after treatment. These [illegible]

continuous vaginal cornification, the animals remained in that condition for from two to fourteen weeks, and then became anoestrous. The weight of the uterus, however, was higher than that in spayed littermate controls, even 26 weeks after treatment. A small amount of oestrogen was clearly being produced throughout the period of observation (as judged by the weight of the uterus) even though it was insufficient to cause cornification in smears, except in the earlier part of the experiment. The vaginal mucosa showed some degree of oestrogenic stimulation, and consisted of more than four layers of cells. Approximately the same results have been obtained on the mouse.

Parkes: That interests me very much because of the work I did years ago with [illegible] of the mouse ovary. At that time we [illegible]

[illegible] activity occurred [illegible]

in vitro. [illegible] findings [illegible]

is, I do not yet know.

Parkes: Then there is a further point; that if you put up the dose of the X-rays too high you destroy the whole ovary in the same way as a smaller dose destroys the oöcytes only.

Krohn: But of course, in the menopausal ovary you have no oöcytes left and no hormone production apparently. The two do go hand in hand there.

Dempsey: In these experiments in which you produce continuous oestrus after irradiation, will the animals mate?

Zuckerman: No. In spite of a completely cornified smear they will not mate.

[illegible] interesting their continuous oestrus [illegible]

[illegible] in which continuous oestrus is produced by constant illumination and in which as long as the animal is maintained under lights, the vaginal smear will be in a cornified

state. Here, however, the continuous oestrus may be interrupted by a variety of devices, by mating or by administration of gonadotrophin or progesterone, in which case the animal will run a cycle or two and then go back into continuous oestrus again. I was wondering if perhaps part of the phenomenon of your animals ceasing ultimately to be in oestrus was not merely a matter of exhaustion. After all, fourteen weeks of continuous oestrogenic secretion in the life of a rat is a long period.

Parkes: In our experience, rats in which cyclic cornification is passing into persistent cornification will quite often mate, and they will certainly mate under conditions where the ovarian tissue they possess does not have any follicular system.

Zuckerman: We specifically tested the animal's readiness to mate, but failed to observe it on any occasion. I do not know why the intermittent cornification in the immediate post-irradiation period fades into con- [illegible]

you do not interfere with the cyclic phase? I was thinking that you might get hypertrophy or overactivity of the adrenal later and this might account for the loss of cornification.

Zuckerman: The experiments on the removal of the adrenals were done when we knew the animals were so many days in continuous oestrus, and from previous experience, could predict they would continue until cornification faded out altogether.

Huggett: What is your index of oestrus?

Zuckerman: We do not call it "oestrus"; we refer to vaginal cornification. I should like to ask Dr. Parkes whether he believes there is any difference between the likelihood of obtaining a successful graft of testicular as compared with ovarian tissue.

Parkes: For the gametogenic property or the endocrine one?

Zuckerman: Gametogenic, although I do not imply the complete formation of spermatozoa.

Parkes: Well, the situation is quite different because unless the testis graft is put in a position where it would normally have a chance of showing spermatogenesis you won't get it, of course.

Zuckerman: I do not mean complete gametogenesis; I mean the multiplication of spermatogonia, and not necessarily spermatocytic differentiation. I was under the impression that it is easier to make a successful graft of testicular than of ovarian tissue, as judged by the continuation of the earlier stages of gametogenesis.

THE HISTORY AND FATE OF REDUNDANT FOLLICLES

P. C. WILLIAMS

Imperial Cancer Research Fund, London

THE fate of redundant follicles, so far as I am capable of dealing with it, is easily summarized. I believe that follicular degeneration or atresia has been observed as a normal happening in the ovary of all mammals that have been studied. It certainly occurs in all laboratory rodents and I am only going to deal with these—primarily with the rat with a side-glance at the guinea pig. Of the many follicles that start on the path of development towards ovulation in these animals, only a small minority reach the goal—most fall by the wayside. It is stated that degeneration may set in at any stage in follicular development, but again I am confining my remarks to atresia of medium-sized and large follicles—broadly, those with an antrum. This is because it is the most striking form of atresia to observe and much the easiest to study. The course of that atresia is familiar.

In the guinea pig, atretic follicles show initially (Fig. 1) a pyknosis of the nucleus, and disintegration, of the granulosa cells bordering the antrum. The granulosa layers rapidly degenerate and, as the follicle shrinks, the inner thecal cells proliferate and take on a fibrocytic appearance (Fig. 2). Meanwhile the ovum also degenerates and finally only the theca retains its structure—it remains unaffected by the secondary degeneration of the fibrocytic tissue, so that thecal nodules (Fig. 3) persist giving the interstitial tissue a nodular appearance before merging into general uniform interstitial tissue (Fig. 4). There is much less fibrocytic proliferation from the theca in the rat, but otherwise the process is exactly the same. In both species, the interstitital cells so constituted.

although retaining an endocrine function as shown by histochemistry (Dempsey and Bassett, 1943; Claesson and Hillarp, 1947; Deane, 1952) and X-irradiation studies (Brambell and Parkes, 1927; Parkes, 1927), are not so prominent as in other species, for example in the rabbit, where they hypertrophy to form an interstitial gland. Thus the fate of the redundant follicle is partly to disappear completely, partly to persist to form part of the endocrine tissue of the ovary.

Now to come to the history of atresia. It has been stated that atresia occurs in cycles in the guinea pig (Aron and Aron, 1953) and mouse (Engle, 1927), being most marked after ovulation in the guinea pig, and during the first day of dioestrus in the mouse, though no such cycle has been proved in the rat (Mandl and Zuckerman, 1950). If there is such a correlation, then it is some evidence that atresia is conditioned by hormonal changes and it is this aspect of the subject I wish to discuss.

First of all I should like to recapitulate some old work on the direct effects of oestrogen on the ovary (Williams, 1944). If an immature rat is hypophysectomized the ovaries atrophy. This atrophy can be at least partly prevented by treatment with oestrogen starting at the time of operation. Table I

Table I

OVARIAN WEIGHT IN IMMATURE RATS AFTER HYPOPHYSECTOMY, OR HYPOPHYSECTOMY AND THE IMPLANTATION OF A STILBOESTROL TABLET

Days after operation	*Weight of ovaries (mg.)*	
	Hypo.	*Hypo.+stilb.*
1	5·9	7·8
2	5·0	8·9
3	6·0	9·2
4	4·6	10·5
5	3·0	8·4
10	3·3	8·7
15	2·9	9·4

5 rats per group. Ovarian weight in 20 normal immature rats = 8·2 mg.

Plate I.

The stages of follicular atresia in the ovary of the pregnant guinea pig (45)

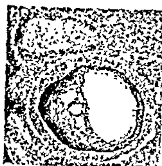


Fig. 1. Earliest and late stage of follicular atresia.



Fig. 2. Intermediate stage of follicular atresia.



Fig. 3. Final stages of follicular atresia: thecal nodules.

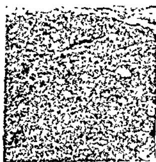


Fig. 4. Merging of the nodules into the general interstitial tissue.

The microscopical preparations were kindly lent by Dr. I. W. Rowlands.

progressively decreases and there is a reduction in the size of the largest follicle.

I have recently studied some of this old material from the point of view of follicular degeneration. Some interesting points arose. The normal immature rat ovary contains about 40–50 follicles more than 200 μ in diameter and of these about half are in the early stages of atresia. My impression is that

Table II

EFFECT OF STILBOESTROL IMPLANTATION AT OPERATION ON THE HISTOLOGY OF THE OVARIES IN IMMATURE, HYPOPHYSECTOMIZED RATS

Animal	*Days after operation*	*Follicles > 200 μ in diameter*		*Mean maximum follicle diameter μ*
		Total	*Solid*	
Normal	—	40	4	390
Hypophysectomized	2	38	5	312
	5	25	8	258
	10	9	6	218
	15	7	7	216
Hypophysectomized and stilboestrol-treated	2	52	16	412
	5	85	53	376
	10	85	48	378
	15	67	38	377

this process is a very rapid one; certainly almost all the follicles in the later stages of atresia are smaller than this, and so are not included in the counts. These is little change in the total count during the first five days after operation but the proportion of atretic follicles is greatly increased. Only 6–10 of the follicles are normal at this time and 20–30 are atretic. The proportion of atretic follicles has increased from 40–50 per cent to 60–80 per cent.

At this point we have to reckon with the observation of Aron and his colleagues (Firket, Petrovich, Marescaux and

Aron, 1953; Aron and Aron, 1953) that a single injection of a crude extract of beef anterior hypophysis produces at a certain dose level atresia of virtually all the follicles in the ovary of the guinea pig. As the dose injected is increased these effects are produced; firstly, follicle stimulation; with a larger dose, atresia; with a still larger dose, luteinization; and with the highest doses, atresia again. Within the limits producing atresia, the higher the dose the quicker the effect. At first sight, this observation seems to conflict with the increase in atresia produced by hypophysectomy in the immature rat—but I am not sure that there is a conflict. I believe that the operation of hypophysectomy releases gonadotrophin into the circulation. The figures in Table I suggest that there is an interruption in the ovarian weight loss on the 3rd day after hypophysectomy. The difference in ovary weight at this point is not statistically significant but I think it is a real one, and Lane and Greep (1935) made a similar observation. There is some supporting evidence for the suggestion that this temporary, slight recovery of ovarian weight is real and a reflection of endogenous gonadotrophin. When a single dose of the gonadotrophin from pregnant mares' serum is given 3–4 days after hypophysectomy to immature rats, the ovarian response is greater than it is if the injection is made before or after this time (Williams, 1945). I think it may well be that gonadotrophin hastens the follicular atresia which certainly occurs, however, in its absence—21 days after hypophysectomy there are still some follicles in the ovary and some of these are undergoing atresia though the proportion (only about 15 per cent) seems to be subnormal.

When we study the ovaries of the oestrogen-treated, hypophysectomized rats there is a different picture. During the first four days after operation there are less atretic follicles than there are in the untreated animal—there are [illegible] atresia.

stimulates the proliferation of the membrana granulosa, but even in the continued presence of oestrogen the degeneration of this tissue is not prevented—it is only delayed for at most several days. I think that there is evidence too that the degeneration may be hastened by gonadotrophin, perhaps acting by the liberation of ovarian androgen. Apart from this, the ageing process in the membrana granulosa is completely obscure. Histochemistry has revealed some interesting facts about the degeneration process once it has started but no-one can yet tell whether a follicle is proceeding to ovulation or will undergo atresia until the degeneration starts.

Postscript

The observation recorded by Professor Zuckerman in his communication suggests to me that all the ovarian tissues are transient. He has certainly convinced me that oögenesis is no longer a function of the ovary in adult life. The transience of the membrana granulosa I have just discussed, and that of the corpus luteum is well established.

Professor Zuckerman's findings in rats with X-irradiated ovaries suggest that the thecal tissue and its successor, the interstitial tissue, are transient too. He has confirmed Dr. Parkes's earlier observation that cyclic vaginal cornification may continue in the complete absence of oöcytes and follicles and has extended this finding by observing that such vaginal cycles do not continue indefinitely. After a number of weeks the vagina reverts to a continuous di-oestrous condition. This suggests that the function of the interstitial tissue is limited in time unless the tissue is continually renewed from the thecal cells of degenerating follicles and corpora lutea. Such a hypothesis would explain the lack of cyclic activity in the postmenopausal woman, in whom, as Professor Krohn pointed out, lack of cyclic ovarian activity is not accompanied by persistence of cyclic vaginal changes.

Acknowledgement.

I am very grateful to Dr. D. J. Trevan, Mr. E. V. Willmott, F.R.P.S., and Mr. G. D. Leach for preparing the photomicrographs.

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DISCUSSION

Amoroso: Before putting Mr. Williams' paper open to discussion I would like to ask him one question. Are these atretic phenomena equally well expressed in the adult ovary as in the immature ovary?

Williams: That I do not know.

[illegible]

luteum.

Rowlands: I can confirm, from my limited experience with the guinea pig ovary, that the egg does seem to be preserved in a good condition for a very long period of time in an atretic follicle and the zona pellucida remains recognizable in a corpus luteum atreticum for a large part of the pregnancy.

Strauss: I would like to ask if you think that antrum formation is dependent on an endocrinological process?

Williams: Yes, I think it certainly is.

night later, or a month later?

Williams: I do not think you can, except from counts. The follicle

I have not tried.

Corner: I have a somewhat unorthodox notion about atresia which

follicles luteinized, and we have seen a number of cases where only a part of the small follicle was luteinized, forming a little partial accessory corpus luteum one side of a tiny follicle. The other side of that follicle was in full atresia.

I have formed the notion that a process of partial atresia, or something very much like it, is an essential step in the conversion of granulosa cells into granulosa lutein cells.

Amoroso: That may also be the case in the ovary of the foetal giraffe where atresia is widespread and is accompanied by the conversion of

atresia that occurs after ovulation in the guinea pig ovary it is my impression that the egg shows changes very rapidly indeed. So, is it possible that we are confusing perhaps two different physiological mechanisms?

Williams: The process of atresia in the oestrogen-treated animal is obscure to me. You don't seem to see the intermediate stages. Unfortunately my material is very scanty between the fifth and the tenth day after hypophysectomy and oestrogen treatment. You do see what are obviously remnants of atretic follicles, which have obviously undergone atresia since treatment started. The general appearance is rather different from those in the normal animal. I think this is a different form of atresia.

Strauss: Is not atresia the normal fate of an egg follicle, and maturation and ovulation only the exception? In humans, for example, the 400 eggs, approximately, which ovulate during the fertile period are unimportant compared with the 400,000 primary follicles. Therefore, there are many more atretic follicles than ripening follicles. What significance do such atresia and atretic follicles have?

Harrison: I was interested to hear you say that you thought granu-

a few days but no longer. Perhaps there is an analogous situation in the ovarian interstitial cells in Prof. Zuckerman's X-irradiated animals. It may be that these cells too have a limited life, and in fact vaginal cornification may cease because there is no continual renewal of interstitial tissue from atretic follicles in the X-irradiated animals.

THE CORPUS LUTEUM OF THE GUINEA PIG

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The salient morphological and histological features of the ovarian changes in the guinea pig have been known for half a century. In 1906, Loeb described its early development and drew attention to the early closure of the rupture point of the follicle, the formation of a fluid-filled central cavity and to the occurrence of haemorrhage before the obliteration of the cavity by luteal tissue. In a later paper, Loeb (1911) gave a comprehensive account of the histology of the ovary during the dioestrous cycle and pregnancy, taking into account the corpus luteum and follicular activity. He noted the greater size of the former during pregnancy and that it remained a large circumscribed organ until parturition. Nevertheless, cytological evidence of regression was obtained which seemed to be associated with loss of vascularity. A rapid and complete degeneration of all vesicular follicles was noted in unmated and pregnant animals for a short period of about four or five days after ovulation, after which follicular growth was resumed, and by the 10th day large healthy follicles together with others showing signs of recent degeneration accompanied the corpora lutea. A number of the healthy follicles soon undergo very decisive changes which are best described in Loeb's own words—"These follicles are characterized by an increase in cytoplasm of the granulosa cells. The nuclei of the granulosa cells are not so densely packed in these follicles as in the ordinary large follicles; this peculiarity being due to the marked development of the cytoplasm. They can easily be recognized in sections stained by haematoxylin and eosin insomuch as they appear stained more reddish in contradistinction to the ordinary large follicles in which the blue colour of the nuclei predominates, while in the mature follicles the red stain of the cytoplasm is a distinguishing feature".

I have confirmed the presence of these large follicles in the guinea pig ovary during pregnancy and their staining reaction, as noted by Loeb, forms a ready means of their distinction from any other follicles that are present. They are, quite clearly, follicles that are undergoing the changes associated with the pre-ovulatory phase of development. In no single case has any evidence of spontaneous ovulation been found during pregnancy. The object of the present work has been to discover if ovulation can be induced during pregnancy and to study the growth of the resulting corpora lutea in the presence of those associated with gestation. However, before embarking on this experimental procedure it was necessary to make a quantitative study of the development of the corpora lutea of the cycle and of pregnancy for, to my knowledge, no work of this sort has been published. The experiments form part of an investigation into the capacity of the guinea pig to maintain a second concurrent pregnancy.

Material and Methods

All the guinea pigs were of the Hartley-Dunkin (M.R.C.) strain, of which eighty were used to establish the normal growth of the corpus luteum of the cycle and after mating with a sterile or fertile male. A vasectomized male was used to effect sterile mating. All observations were dated from the post-partum oestrus to ensure that every animal was fertile and that each one contributed to the replenishment of the stock before being put to experimental use. The times of parturition and post-partum mating were accurately recorded for those animals used to obtain early developmental stages of the corpus luteum, but for later stages the occurrence of mating was presumed by the discovery of a vaginal plug or by sperm in a vaginal smear.

Chorionic gonadotrophin, used to induce ovulation, was injected into one of the small ear veins and the result observed by examination of tubal washings for eggs or by the serial section of the Fallopian tubes. The ovaries and the reproductive tract were fixed in Bouin's fluid overnight and serial

sections cut at 7 μ; every 5th section was mounted and stained in haematoxylin and eosin.

Measurements of the corpora lutea and the mature follicles were made across three diameters. One of these was got by counting the number of sections in which the organ appeared and the other two by measuring with a micrometer eyepiece the largest section of the organ; measurements were made at right angles through the centre of the section. The product of the three diameters in mm.^{3} (D^3) has been used throughout to express the dimension of these organs from which true volume may easily be calculated.

The Corpus Luteum

Quantitative observations

At ovulation, which occurs about fifteen hours after parturition, the mature follicle measures about 1 mm. in diameter, by which time changes suggestive of luteinization have already commenced in the membrana granulosa. Fig. 1 gives the mean

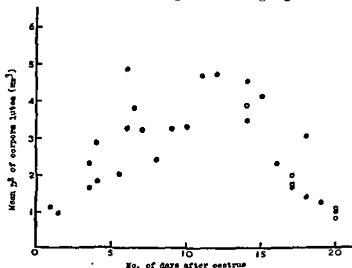


FIG. 1. Mean D^3 in mm.^{3} of the corpora lutea of the guinea pig (●) and after sterile mating (0).

Fig. 2. Mean D^3 in mm.^{3} of the corpora lutea of pregnant guinea pigs.

Fig. 3. Regression lines and formulae of data given in Figs. 1 and 2.

D[3] of the corpora lutea in 25 unmated guinea pigs and in five which were mated to a sterile male, and Fig. 2 is a similar representation of the same organs in fifty pregnant guinea pigs. The regression lines calculated from the data are given in Fig. 3.

The growth rate of the corpora lutea in mated and unmated animals is very similar but the period of growth differs. In the latter it ceases on about the 12th day and the organs then rapidly regress; the regression is not inhibited as the result of sterile mating. In the pregnant animal the corpus luteum continues to grow until the 18th to 20th day and is maintained at this size for the rest of pregnancy, but after parturition it rapidly becomes disorganized. Its involution occurs when the new corpora lutea are actively growing in the same ovaries.

Microscopic observations

This investigation is not primarily concerned with detailed cytological changes in the corpus luteum, but a general description of the more gross histological features of the ageing organ is required since it is on these grounds that the distinction between the normal and the induced corpora lutea is made. The mature follicle protrudes only slightly above the surface of the ovary and after its rupture forms only a small crater over which the germinal epithelium is re-established in 48 to 72 hours. Both the granulosa and the theca interna contribute to the luteal tissue which at three to four days consists of a narrow band enclosing a central cavity as described by Loeb. The cells are small and fairly closely packed; they have a small dark-staining nucleus surrounded by clear cytoplasm of irregular shape. Mitotic figures are not common at this stage. Possibly because of compression set up in the cavity by the ingrowth of luteal tissue, haemorrhage occurs very commonly and in some of the corpora lutea the cavity becomes packed with red blood cells for as long as the 20th day in some pregnant animals, but in others where bleeding is less severe strands of connective tissue encroach and obliterate the cavity by the 7th day or soon afterwards. The luteal cells

meanwhile expand; the cytoplasm enlarges and the nucleus becomes spherical in shape. At this stage the corpus luteum has a good blood supply. Changes up to the 12th day are approximately similar in unmated and mated animals, but afterwards, the corpora lutea in the former gradually undergo regressive changes which become intensified when ovulation recurs on the 16th to the 18th day.

Meanwhile in the pregnant animal, the corpus luteum shows little change until after the 20th day when the luteal cells gradually enlarge and tend to a polyhedral shape. Mitoses are seen more commonly and the gland has a very rich blood supply. This condition of apparent high activity persists until about the 35th day when a very gradual ageing process sets in; the outline of the cells becomes less distinct, the cytoplasm is more coarsely granular and vacuoles appear, and the nucleus becomes elliptical in shape. Nevertheless, a small number of the luteal cells appear which have all the characters of those in a young, functional corpus luteum. The amount of fibrous connective tissue increases, which probably serves to maintain the size and shape of the gland. The most striking change which occurs over this period is the restriction of the blood supply due to a progressive closure of the vessels.

The gland as a whole, however, remains circumscribed until parturition after which it collapses. In so far as a similar change occurs in the corpus luteum of the unmated guinea pig when a new set of follicles rupture, it may well be that the final collapse of the gland at parturition is associated with the recurrence of ovulation at the next oestrus, fifteen to eighteen hours later, rather than from the withdrawal of placental hormones or the evacuation of the uterus.

Follicular Activity and the Induction of Ovulation in Pregnancy

At the time of post-partum mating, all follicles other than those destined to ovulate twelve hours later are undergoing atresia, and for the four days after rupture no healthy

vesicular follicles are to be found. But as Loeb noted, a new wave of follicular growth then takes place and by the 8th to 10th days mature follicles are present, and in the series of ovaries examined at least one of these structures was found throughout pregnancy. No evidence of spontaneous ovulation was obtained.

A few initial experiments indicated that a dose of 50 i.u of chorionic gonadotrophin was sufficient to induce ovulation in pregnancy. A summary of the results obtained in 33 guinea pigs, given in Table I, indicates that the follicles in early

Table I

The Frequency of Ovulation Induced by an Intravenous Injection of 50 i.u. of Chorionic Gonadotrophin at Different Stages of Pregnancy

Injection period (days)	*No. of animals injected*	*No. of animals ovulating*	*Average no. of follicles ruptured*
8–20	10	5	2·0
21–40	16	16	2·8
41–62	7	7	3·9

pregnancy (8 to 20 days) are less sensitive to the action of the hormone than are those occurring in late pregnancy, when the average number of follicles that rupture is nearly doubled.

The Fate of the Ruptured Follicle

Guinea pigs were injected with chorionic gonadotrophin on the 8th, 20th and 35th days of pregnancy; they were killed 8, 14, 20 and 28 days later and the induced and the normal corpora lutea were measured as described above. Classification of the two sets of corpora lutea was based on qualitative rather than quantitative differences shown. By these means the induced corpora lutea at 8, 14 and 20 days of age are readily distinguishable from those of pregnancy, but in some ovaries the differences between the 28-day-old induced corpora lutea

and the aged corpora lutea of pregnancy are not great. Although they may be different in size it was felt that this criterion was no certain means of classification, and to enable the writer to be unaware of their size at the time of histological examination, he was shown only a high-power microscopic

Table 2

THE GROWTH OF CORPORA LUTEA INDUCED AT DIFFERENT STAGES IN PREGNANCY

Code No. IOP.	*Induction of ovulation in pregnancy (day)*	*Age of induced corpora lutea (days)*	*Stage in pregnancy (days)*	*No. of corpora lutea*		*Mean D^3 (mm³) of corpora lutea*	
				Pregnancy	*Induced*	*Pregnancy*	*Induced*
11	8	8	16	7	1	4·96	1·62
13	8	8	16	4	2	6·01	3·05
9	8	14	22	6	2	6·72	2·10
10	8	14	22	6	1	5·63	2·32
12	8	20	28	2	2	6·28	1·94
14	8	20	28	4	4	7·55	2·57
21	21	8	29	8	3	5·41	1·49
23	21	8	29	9	1	5·89	2·26
28	21	14	35	5	2	6·34	3·57
24	21	14	35	6	3	4·94	2·56
26	21	20	41	6	2	4·95	3·37
22	21	20	41	6	4	6·01	3·53
25	21	28	49	7	1	7·00	4·93
27	21	28	49	6	7	5·21	2·96
17	35	8	43	9	3	6·98	3·03
15	35	8	43	4	4	9·28	2·64
18	35	14	49	5	8	8·20	4·19
31	35	14	49	5	5	6·69	2·89
16	35	20	55	6	3	9·69	4·63
19	35	20	55	6	1	5·88	4·39
30	35	28	63	5	4	8·39	3·89
20	35	28	63	10	4	7·31	4·38

field upon which classification was based. A very close agreement between classification based on the results of microscopic examination and size of the gland was obtained. The most reliable criterion used to separate the two sets of glands was the degree of vascularity but when at a later stage of development closure of the blood vessels had occurred in the

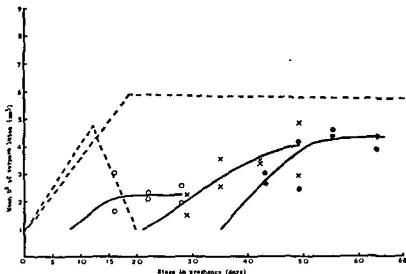


FIG. 4. Mean D³ in mm.³ of corpora lutea induced on the 8th (O); 21st (×) and 35th day (●) of pregnancy compared with the normal structures in unmated and pregnant guinea pigs.

induced corpora lutea as well, the distinction was made on cell size, nuclear shape, vacuolation of the cytoplasm and the estimates of the numbers of mitotic figures present. The data obtained are presented in Table 2 and Fig. 4 on which lines fitted by eye, illustrate the trend of growth of the corpora lutea induced at the three different stages of pregnancy. The first wave of follicles to mature, when ruptured artificially on the 8th day of pregnancy, form very small corpora lutea growing at a very slow rate, but although they do not attain the size of those in the unmated animal, they are maintained

at their full size for a longer period. It would seem that the later in pregnancy that ovulation is induced, the more rapid is the growth and the greater is the size attained by the corpora lutea. The follicles ruptured on the 35th day form corpora lutea of similar size to those produced during the di-oestrous cycle but unlike the latter they remain at their maximum size, as do the corpora lutea of pregnancy. Histologically, the induced corpora lutea resemble those of the unmated animal for they do not undergo the changes characteristic of normal structures during the 3rd to the 4th week of pregnancy.

The corpora lutea of pregnancy in the ovaries of the guinea pig in which ovulation was induced on the 35th day (mean $D^3 = 7 \cdot 70 \pm 0 \cdot 68$ mm.^{3}) were significantly larger than those (mean $D^3 = 5 \cdot 65 \pm 0 \cdot 37$ mm.^{3}) in ovaries containing a set of corpora lutea induced on the 21st day. Enlargement does not seem to be related to the number of induced corpora lutea co-inhabiting the ovaries, although in one animal (IOP/19) it did not occur when only one of the latter structures was present.

Functional Activity of the Induced Corpora Lutea

The inhibition of follicular activity

Six pregnant guinea pigs were injected with chorionic gonadotrophin and killed 24 to 96 hours later. All follicles present at the time of injection which failed to ovulate became heavily luteinized and were recognizable as small luteal cysts containing an egg. No normal vesicular follicles were found in ovaries up to 96 hours after injection, but in other guinea pigs killed after eight days they were as plentiful as in a normal animal after mating.

Capacity to sensitize the uterus to decidual reaction

A few attempts were made to test the capacity of induced corpora lutea to sensitize the uterus in this way. Unilateral pregnancy was established by ligation of the left uterine horn

or the left Fallopian tube in eight young guinea pigs, and at various stages after post-partum oestrus they were injected with chorionic gonadotrophin. Six days later silk threads were inserted through the sterile horn and the sites examined histologically six days afterwards. No evidence of a decidual reaction was obtained but in two animals in which ovulation was induced on the 53rd day of pregnancy the infertile horn was enlarged around the site of irritation. It should be realized that the horn sterilized by ligation at its cervical end is a large flaccid structure containing a fluid and bears no resemblance to the uterus in the early luteal stage. The latter condition was more closely obtained in animals that were semi-sterilized by ligation of the Fallopian tube, but in two of the eight animals treated in this way there was no sign of a decidual reaction.

Effect on parturition

Two guinea pigs were injected in very late pregnancy to observe the effects of the presence of induced corpora lutea on parturition. One, which was treated on the 54th day, gave birth to a litter at the expected time, fourteen days later. No evidence of post-partum mating was obtained but when killed three days afterwards the ovaries contained eleven very young corpora lutea. The second animal, injected on the 62nd day, produced a litter seven days later. She was killed six hours after parturition and the ovaries, which contained six induced corpora lutea, also had numerous mature follicles awaiting ovulation. There is little to suggest, therefore, that the induced corpora lutea affect the time of onset of parturition or the recurrence of post-partum ovulation.

Discussion

The corpus luteum is an endocrine organ whose main function in unmated animals is the regulation of the dioestrous cycle by means of its capacity to inhibit ovulation. Of all the structures in the body, it is probably the one which has the shortest life, for in normal circumstances it remains

active for periods ranging from a few days to about two weeks according to the species. Its life becomes extended in the process of reproduction and it assumes important functions in the maintenance of gestation. The extent to which it is prolonged and its relative importance to the needs of gestation varies greatly from one species to another. Reynolds (1949), in summarizing the evidence for the rôle of the corpus luteum in the maintenance of pregnancy in different animals, places the guinea pig in an intermediate position between those species in which luteal tissue is required for foetal survival and those in which it plays no part in the second-half of pregnancy, for the reason that abortion does not invariably occur after oöphorectomy in late pregnancy. The observations reported above have shown quite clearly that the corpora lutea persist as well-organized bodies throughout pregnancy in the guinea pig. But mere existence does not necessarily imply retention of functional activity, and in this connection reference must be made to the work of Loeb and Hesselberg (1917) showing that pregnancy was initiated and maintained in some guinea pigs for as long as thirteen days after oöphorectomy performed as early as the 3rd to 6th day after mating. In others, treated similarly, abortion occurred. That some fundamental change in the function of the corpus luteum takes place at this very early stage of pregnancy is suggested by the renewal of follicular growth and maturation in the guinea pig ovary on about the 4th to 5th day after ovulation. It is difficult in this species, therefore, to establish the precise relationship between the corpus luteum and the maintenance of gestation.

It would seem, in general, that there are two factors closely associated with the prolongation of the life of the corpus luteum. First, in some species, for example the rat, the duration of luteal activity is prolonged by cervical stimulation at mating. In the guinea pig, however, it has been shown above that mating with a vasectomized male fails to prevent the regression of the corpus luteum which normally occurs on the 12th day after ovulation. Secondly, many lines of enquiry

suggest that luteal growth is stimulated at the time of implantation of the blastocyst. This is shown by many species which exhibit the phenomenon of delayed implantation, for example many species of the *Mustelidae* (Wright, 1942) and the *Pinnipedia* (Harrison, Harrison Matthews and Roberts 1952; Rand 1955), and in species in which implantation is delayed by concurrent lactation, for instance the bank vole (*Clethriomys glareolus*) examined by Brambell and Rowlands (1936). In all these there is evidence that the growth rate of the corpus luteum slows down or may even stop when the blastocyst is in the free-living state in the uterus, but when attachment takes place luteal growth is resumed. Experimental evidence of the rôle of the uterus in prolonging luteal activity has been provided by Nalbandov, Moore and Norton (1955), who have shown that distension of the uterus of sheep by insertion of a bead 8 mm. in diameter delayed the onset of the next oestrus by about eight days. In the sheep, however, no difference in size exists between the corpus luteum of the cycle and that in the pregnant animal, so that it is not possible to show whether the uterine stimulus is able to enlarge as well as to prolong the period of its activity. Clearly, an experiment of this nature is desirable in the guinea pig.

Attachment of the blastocyst to the uterine epithelium in the guinea pig occurs on the 6th day after fertilization (Blandau, 1949) at which time the uterus is sensitive to stimuli producing the decidual reaction. There is, however, no suggestion in Fig. 2 of a change in the growth rate of the corpus luteum at this time and if attachment is responsible for the continuation of luteal growth from the 12th to the 20th days of pregnancy (over which period the corpus luteum is regressing in the non-pregnant animal) it becomes clear that the effects of the stimulus are not apparent for some time later. It would seem probable on the basis of the work of Velardo and colleagues (1953) on the quantitative relationship between the decidual response and extension of luteal function in the rat, and from Nalbandov's experiments on sheep (*loc. cit.*), that the stimulus for the continued growth of the corpus luteum of pregnancy

is provided only when a certain threshold amount of decidual development or uterine distension has occurred.

It has been made clear that not only is the life of the corpus luteum prolonged in the pregnant guinea pig but that it is also significantly enlarged. Are these two features different expressions of a response to the same stimulus or are they controlled independently? It is well known that in many species prolongation of luteal activity is not accompanied by enlargement of the gland, which may be an indication that these characters are controlled separately. The observations made on a set of corpora lutea resulting from ovulation induced at different stages during pregnancy confirm this view for, although these structures never attain the size of the normal corpora lutea of pregnancy, they have a longer life than those of the unmated animal.

Summary

The corpus luteum of the guinea pig reaches maximum size on about the 12th day and regression, which sets in immediately, is accelerated when ovulation recurs four to six days later. Sterile mating does not prolong its life-span. In pregnancy, the corpus luteum grows at the same rate for eighteen to twenty days and is maintained at maximum size until parturition, but histological evidence of ageing occurs in mid-pregnancy. Regression is very rapid after parturition.

Follicular growth is resumed on the 4th to 5th day after mating and mature follicles are found at all times after the 8th or 10th days. Ovulation does not occur spontaneously but may be induced by chorionic gonadotrophin (50 i.u.) injected at any time after the 8th day. The number of follicles that rupture and the size of the corpora lutea that are induced are greater in late than in early pregnancy. Those produced on the 53th day grow to the size of the corpora lutea in the unmated animal. They never reach the size of the co-existing corpora lutea of pregnancy but, like the latter, they are

maintained at their full size for a long time. The corpora lutea of pregnancy are enlarged when they co-inhabit ovaries containing another set induced on the 35th day, but not when ovulation was induced on the 21st day.

Follicular growth is inhibited for four to five days following the induction of ovulation in pregnancy. The induced corpora lutea are incapable of sensitizing the sterile horn of unilaterally pregnant guinea pigs, and their presence in very late pregnancy does not interfere with parturition.

A factor associated with decidual growth is considered to be responsible for the additional growth occurring in the corpus luteum of pregnancy and which is different from that maintaining the size of the corpus luteum until parturition.

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DISCUSSION

[illegible] if he has done any histo-

Rowlands: No histochemical investigations have been made, but with the limited number of ovaries examined by ordinary histological methods there is nothing to suggest that the early development of the induced corpus luteum is abnormal.

Harrison: Do you think they function, do they produce anything?

Rowlands: I think it would be most difficult to prove their functional [illegible]. In two animals in which ovulation was induced very late on in [illegible] corpora lutea had no effect on the occurrence [illegible]ately afterwards, at the same time [illegible] The other possibility is to observe [illegible] growth, but the latter recurs so [illegible] the guinea pig that little time is [illegible] their capacity to [illegible] rapidly after normal ovulation in the guinea pig that little time is available for observations to be made. Certainly, for the first 4–6 days after induced ovulation and in the presence of induced corpora lutea, vesicular follicles are absent. But at eight days after induced ovulation they have reappeared. To this extent, there is some slight evidence that they are functional.

Krohn: Do I understand correctly that you cannot do in the guinea pig what you can do in the rabbit—prolong the duration of pregnancy by inducing a new set of corpora lutea?

Rowlands: No. In the two animals in which I induced ovulation very late on in pregnancy, so that at the time of expected parturition the [illegible] lutea would be in a fairly active state, they did not [illegible] stages of pregnancy [illegible] [illegible]culiar animal in [illegible] is not quantal, [illegible] this treatment.

Rowlands: The [illegible] effect of ovariectomy [illegible] in this respect for the effect of ovariectomy [illegible] that is, abortion does not occur in all animals after this treatment. Loeb and Hesselberg, many years ago, claimed that the corpora lutea are required only for about the first 6 days after mating, but that ovariectomy on the 3rd to 5th days of pregnancy caused some to abort on about the 12th day.

Jost: This has been studied again recently in Prof. Courrier's laboratory by Artunkal and Colonge in 1949. They found that castrating the guinea pig before day 16 always produces abortion, but this abortion may be avoided by progesterone. Later castration does not induce abortion. So this is good evidence that the corpus luteum is necessary during the first 16 days of pregnancy but afterwards it may be suppressed.

Huggett: Of course, if the corpus luteum is persistent the placenta may not have to do quite so much. They may be supplementing each other.

T.-Duplessis: Do you have any data concerning the growth of the corpora lutea during delayed pregnancy, for example in lactating rats or other animals?

Rowlands: Not in the guinea pig but in the bank vole (*Clethriomys glareolus*) there is a slow initial growth phase when the egg is free in the tube and in the uterine lumen, which is followed by a second burst of growth following the stimulus provided by implantation.

T.-Duplessis: It would be interesting to know what happens in animals like the mink where I believe you have delayed pregnancy of about four or five months.

Rowlands: I have no information to offer about mink, but in various species of seals in which implantation is also delayed for some months after mating, Prof. Harrison has described changes in the corpus luteum coincident with implantation.

Harrison: My impression was, in examining that material, that the corpus luteum of ovulation, if you like to call it that, was not properly vascularized, and that at the time of implantation, perhaps some two or three months later, the corpus luteum became revascularized, and that before implantation there was an outbreak of vacuolation of the luteal cells, which went when implantation occurred.

Matthews: Something similar seems to occur in the badger also.

Amoroso: With regard to the persistence of corpora lutea, the cat [illegible]

is actually entering the endometrium.

Accessory corpora lutea, such as are found in the mare, are sometimes encountered in the ovary of pregnant cats during the seventh week of gestation.

Formation of the acrosome and head cap

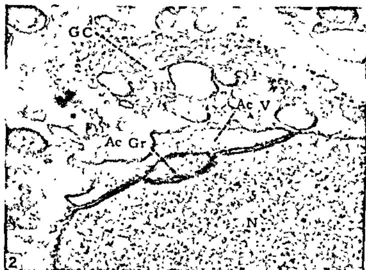
The large juxtanuclear body of the spermatid which is commonly referred to as the *idiosome-Golgi complex*, appears under the electron microscope as an aggregation of minute vacuoles and broad, flattened vesicles (Figs. 2 and 3). The latter are often closely approximated in parallel array and hence, in section, have a lamellar appearance. The small vacuoles seem to arise by being budded off from the edges of the flattened vesicles. With cytological staining methods, the parallel arrays of membranes are apparently impregnated more heavily with osmium or silver than is the rest of the complex and they are identified with the light microscope as rod-like or crescent-shaped Golgi bodies (dictyosomes). The masses of minute vacuoles, staining less heavily, have been designated the *idiosomal material* (*archoplasm*). Inasmuch as the Golgi complex in all other cell types examined to date also contains both the minute vacuoles and the parallel arrangements of membrane-bounded vesicles, there appears to be no reason for retaining, in the case of the spermatid, the special terms "idiosome", "archoplasm" or "sphere" for a part of this structure. Instead, the whole body is to be regarded as a particularly well-developed Golgi complex, differing in no essential respect from that of other cell types.

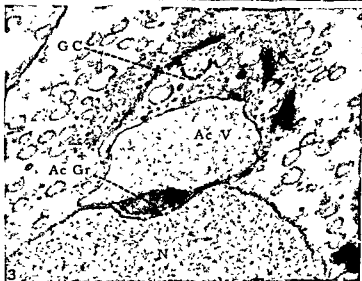
Early in the differentiation of the spermatid one or two large granules are formed within separate vacuoles of the Golgi complex. These proacrosomal granules are moderately dense to electrons and are generally homogeneous with present resolutions. Coalescence of the vacuoles, and of the granules which they contain, results in the formation of a single sizeable acrosomal granule within a rather large acrosomal vesicle. The latter is bounded by a distinct membrane and, in life, probably has a fluid content but this is represented in the electron micrographs only by a faint, flocculent precipitate seen in the vacuole around the acrosomal granule (Fig. 3). The acrosomal vesicle approaches the nucleus and adheres to its anterior pole and at the same time the granule in its



FIG. 1. A pair of spermatids (Spd) joined by an intercellular bridge (at arrow) through which there is free communication from one cell to the other. The spermatids are completely surrounded by Sertoli cells (Se C).

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progressive enlargement.



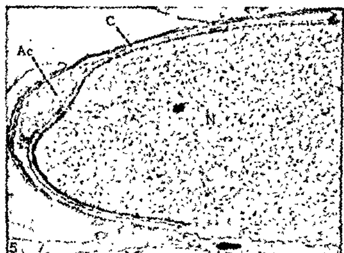


Fig. 4. The acrosomal vesicle (Ac V) has spread out over the nucleus

half of the nucleus to form the head cap (C). Its lumen is reduced to a narrow cleft and its content is considerably more dense than before. The acrosome granule (Ac Gr) is markedly flattened.





Fig. 6. The head of a spermatid in a slightly more advanced stage

interior becomes fixed to that portion of the wall of the vesicle that is attached to the nuclear membrane. The Golgi complex thereafter remains closely applied to the surface of the acrosomal vesicle (Fig. 3) and gives rise to numerous vacuoles which are believed to coalesce with the vesicle, contributing in this way to its progressive enlargement. The acrosomal granule also grows, seemingly, by addition of material to its surface from the surrounding fluid. The granule at first is almost spherical in shape and projects well into the vesicle, but later it becomes flattened and frequently occupies a shallow depression in the end of the nucleus (Figs. 2 and 3). As the area of contact between the enlarging acrosomal vesicle and the nuclear membrane increases, a layer of dense granular material is deposited on the inner aspect of that part of the nuclear membrane which is covered by the vesicle (Figs. 4, 5 and 6). The vesicle gradually extends down over the end of the nucleus like a double-layered stocking cap and, as it does so, its lumen is reduced to a narrow cleft (Figs. 4 and 5). Meanwhile, in the elongation of the spermatid, the Golgi complex and the associated cytoplasm migrate to the posterior pole of the nucleus and the plasma membrane at the anterior end of the cell is brought into close contact with the outer membranous wall of the acrosomal vesicle or head cap. The substance of the granule, which originally was con-

this stage, therefore, consists of outer and inner membranes which are continuous at the posterior margin of the cap, and between these is a thin layer of homogeneous material derived from the substance of the acrosomal granule (Figs. 6 and 7).

Thus, the study of electron micrographs has clarified the nature of the idiosome and has reaffirmed the origin of the acrosome from the Golgi complex; it has established that the acrosomal vacuole or vesicle is not a fixation artifact but is a real structure which has an important rôle in the formation of the head cap of the spermatozoön.

Condensation of the karyoplasm

After formation of the head cap a sequence of striking changes is observed in the nucleus. Throughout the early stages of differentiation, the karyoplasm of the spermatid consists of closely-packed fine granules (circa 100 Å) of rather low density, interrupted here and there by coarser granules of somewhat greater density (Figs. 4 and 5). The nucleolus is inconspicuous and indefinite in outline. During the period of elongation of the nucleus, the fine granules in the karyoplasm are gradually replaced by coarser granules. These probably arise by agglomeration of the pre-existing fine granules. However, more seems to be involved than a change in the state of aggregation of the nuclear material for, associated with the increase in granule size, there is a marked increase in their osmiophilia (Fig. 7). This must reflect an increase in the number of unsaturated linkages. As differentiation continues and the coarse granules become more closely packed, some sperm heads show sizeable clear areas of irregular shape which, no doubt, correspond to the "vacuoles" that have been observed with the light microscope in the heads of human spermatozoa. These have not been seen in the other species studied. Late in the maturation of the spermatids, the coarse dense granules (300–400 Å) coalesce into a smooth homogeneous mass which once again displays only a moderate affinity for osmium. Concurrently with these changes in the fine structure of the karyoplasm there is a steady reduction in the size of the nucleus and a change in the shape of its sagittal section from obtuse to acuminate.

The electron microscope finding, that there is a waxing and waning of the osmiophilia of the nucleus, is reminiscent of the earlier reports in the literature to the effect that there is a [illegible] spermatozoa are maturing.

The increase in intensity of nuclear staining in the course of

spermatogenesis has generally been looked upon as the result of a concentration or dehydration of the karyoplasm. The electron microscope observations reported here suggest that the process is not as simple as this and that it involves not only profound alterations in the state of dispersion of the nuclear chromatin but, very likely, changes in its chemistry as well.

Observations on the interstitial cells

Appearance with the light microscope

Human interstitial cells occur as groups of elongated, rounded and polyhedral cells of diverse cytological character. The marked differences in their appearance are interpreted by some investigators as representing different phases in the life-cycle of the Leydig cells (Hooker, 1944; Williams, 1950). Since mitotic figures are not observed in mature Leydig cells, it is commonly assumed that they arise by differentiation from spindle cells present in the interstitium, but it remains unsettled as to whether these cells of origin are common connective tissue fibroblasts, or whether they are primitive *mesenchymal cells which persist in the adult along the blood* vessels and in the lamina propria of the seminiferous tubules.

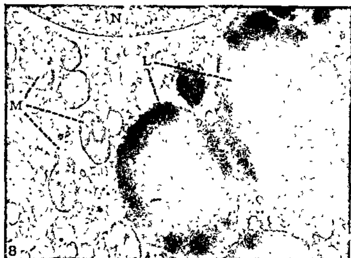
When the young interstitial cells are still relatively undifferentiated, they have a plump, fusiform shape with a nucleus of infolded or irregular outline, a finely granular chromatin pattern and a small nucleolus. Although they resemble fibroblasts in some respects, they are generally larger, often contain a few lipid droplets (Montagna, 1952) and, with some fixatives, their cytoplasm has a fibrillar texture (Sniffen, 1950). In the course of their metamorphosis into typical Leydig cells, the nucleus is said to become round in contour, eccentric in position and to develop a very distinct nuclear membrane and a large nucleolus. The cell volume increases and the cytoplasmic filaments gradually give way to fine acidophilic granules. In the mature Leydig cell, the cytoplasm becomes increasingly heterogeneous and contains

coarse, as well as fine, acidophilic granules, golden-brown lipochrome pigment and lipid droplets of various sizes. In addition, many of these cells contain large angular inclusions known as the crystalloids of Reinke (Reinke, 1896). These are visible in the cytoplasm of unfixed interstitial cells as highly refractile, pale yellow bodies which are usually rod-like, rectangular or trapezoidal in shape, but are often rounded at the ends. They are peculiar to the human testis, and their origin, chemical composition and physiological significance are unknown.

Appearance with the electron microscope

The electron microscope reveals an even greater variation in the fine structure of the interstitial cells than was appreciated with the light microscope, and provides additional evidence of a transition from spindle cells to Leydig cells. The spindle cells in the lamina propria of the seminiferous tubules have elongated nuclei and a homogeneous cytoplasmic matrix of low density to electrons. Their cytoplasm is traversed by a few canalicular strands of ergastoplasm which show vesicular dilations along their length. Mitochondria are few in number and simple in their internal structure. The only cytoplasmic inclusions observed are a few small lipid droplets.

The larger fusiform cells that are found along the blood vessels and interspersed with the Leydig cells have round or indented nuclei and their cytoplasm is characterized by the presence of large numbers of extremely thin (50 Å) filaments of indefinite length (Fig. 10). These are most abundant in the interior of the tapering cell processes where they generally run parallel to the long axis of the cell. The mitochondria and endoplasmic reticulum tend to be near the periphery of the cell. Many small vesicles (300–500 Å) are found just beneath the plasma membrane and these sometimes appear to open at the cell surface. Some of these cells contain sizeable agglomerations of dense osmiophilic granules clustered around vacuoles of lower density.



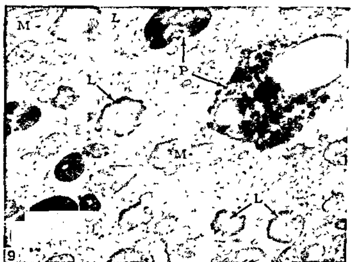
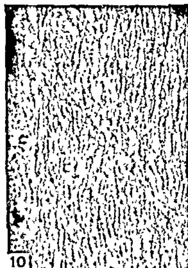


FIG. 8. A portion of a Leydig cell, including a little of the nucleus (N) [illegible]







cells
rs of
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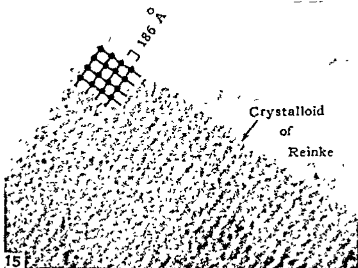


Fig. 14. Electron micrograph of a segment of a crystalloid of Reinke (Cr) disclosing a highly ordered internal structure which presents a fabric-like pattern.

Fig. 15. The end of a crystalloid of Reinke at high magnification showing a pattern of densities spaced a uniform distance apart (*186 Å*) *in precise rows intersecting at approximately a right angle.* This pattern is interpreted in the diagrammatic overlay as the orderly arrangement of protein macromolecules in a crystalline lattice.

The mature Leydig cells are epithelioid in shape but their membranes are not closely coherent as in epithelial tissues. The nuclei are round and have prominent nucleoli. The cytoplasm is not fibrillar, but, instead, has a vesicular character, owing to the presence of very numerous small membrane-limited vacuoles 100 to 200 millimicrons in diameter (Fig. 11). Some of these seem to have a content of very low density but the majority appear quite empty. Their origin and significance is by no means clear. Their membranous wall is sometimes observed to be continuous with the plasma membrane in a manner which suggests that they either are formed at the cell surface or discharge their contents there. In some Leydig cells these minute vesicles are present in moderate numbers, while in others they are so closely packed that little background cytoplasm can be seen between them. It is easy to speculate that they are somehow related to the specific function of the Leydig cells and that the variations in their abundance from cell to cell reflect different states of physiological activity.

The mitochondria are not particularly abundant in Leydig cells and are quite variable in shape. Some are elongated, some spherical, and others have a bizarre, swollen appearance. This pleomorphism is consistent with reports, based upon light microscopy, that the mitochondria of interstitial cells are especially labile and very apt to assume enlarged and distorted forms as a result of delayed fixation (Duesberg, 1918). The mitochondrial matrix is of very low density and the folds of the membrane projecting into the interior of the organelle are fewer and more irregular in their distribution than in the mitochondria of most other tissues. In keeping with the acidophilic staining of these cells in ordinary histological preparations, the endoplasmic reticulum or ergastoplasm is poorly developed and the small particles of ribonucleoprotein, which are responsible for cytoplasmic basophilia (Palade, 1955), are present in small numbers.

Lipid occurs in the Leydig cells in two forms: homogeneous, moderately osmiophilic droplets (Fig. 8), and heterogeneous

aggregates of intensely osmiophilic, granular material (Fig. 9). The former are interpreted as droplets of neutral fat, while the latter are believed to belong to the group of acetone-insoluble lipid complexes often referred to as lipochrome pigments or ceroid. The intensely osmiophilic granular material is often aggregated around the periphery of vacuoles of a homogeneous substance which does not reduce osmium. Sharp angular clefts which sometimes occur in the midst of these conglomerations are interpreted as negative images of crystals which have been dissolved out during specimen preparation. The significance of these pigment masses is not clear, but they are of very common occurrence in the cells of most steroid-producing endocrine glands. The amount of lipid present in the mature Leydig cells is highly variable. Some of them are virtually devoid of droplets of neutral fat but contain considerable pigment. Others have large amounts of both.

In electron micrographs, the crystalloids of Reinke have a complex and highly-ordered internal structure which presents a regular pattern bearing a superficial resemblance to that of a woven fabric (Fig. 14). The pattern varies, depending upon how the object is cut, but in crystals which are most favourably oriented with respect to the plane of section, it is possible to resolve a pattern of densities 100–150 Å in diameter, uniformly spaced about 190 Å apart along two axes which are approximately at right angles to one another (Fig. 15). The length of the period along the third axis has not been determined. This pattern is thought to represent the arrangement of macromolecules in the lattice of a protein crystal. A few of the crystals are rectilinear, with straight sides and sharp angles, but the majority have rounded contours, particularly at the ends (Fig. 13). There are indications that they are almost as soft as the surrounding cytoplasm for, when sectioned, they cut perfectly smoothly without tearing or setting up vibrations in the block. Protein crystals are apt to be rounded at the ends rather than sharp-angled and, in the "wet" state, they are quite soft.

Heretofore, the assumption that the crystalloids of Reinke are protein has been based entirely upon their solubilities and staining reactions as studied with the light microscope. The present study presents additional evidence of their protein nature in their rounded shapes and soft consistency, and it provides the first visual demonstration of their large molecular size and crystalline internal organization. It is apparent that the crystals of Reinke are entirely different from the crystalloids of Spangaro and Lubarsch which are found in the epithelium of the seminiferous tubules.

In addition to the spindle-shaped interstitial cells and the typical Leydig cells just described, many cells are observed which appear to be intermediate between these two cell types. These transitional forms have some of the cytological characteristics of both. Thus, one may find bundles of delicate filaments, typical of the spindle-shaped interstitial cells, intermingled with numerous sub-microscopic vacuoles of the sort commonly found in mature Leydig cells (Fig. 12). The occurrence of such cells seems to support the contention of earlier workers who postulated a gradual metamorphosis of the spindle cells into mature Leydig cells. Moreover, the striking variability among mature interstitial cells with respect to the degree of vacuolation of their cytoplasm and the abundance of their lipid droplets, pigment granules and protein crystals, strongly suggests that they are of very different ages or that they are in different phases of a cycle of physiological activity. There seems to us to be little to support the contention that the Leydig cells clear themselves of these accumulated inclusions and revert to spindle cells (Rasmussen, 1932; Williams, 1950), but there is some reason to suspect that they age and ultimately degenerate. Certainly many cells are seen which are crowded with lipid and pigment and have grossly swollen, distorted mitochondria, and some of these also show large angular areas which, at high magnification, are pale, poorly defined and devoid of visible internal structure, as though they were "ghosts" of crystals which had undergone dissolution. Although dependable criteria for the

recognition of senility in cells at the electron microscope level have not yet been established, it is difficult to escape the conclusion that some of the Leydig cells are maturing while others are no longer physiologically active and are, perhaps, in a state of impending degeneration.

Acknowledgement.

We are greatly indebted to Dr. Fred A. Simmons of the Department of Gynecology of Harvard Medical School for making available to us the human testicular biopsies upon which this study is based.

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DISCUSSION

Montagna: I have scarcely recovered from the breath-taking beauty of these preparations. Before I make any comments (which unfortunately have very little to do with Dr. Fawcett's presentation) may I say that I have been accused of dealing only with the testes of feeble-minded individuals. This is largely true because they are the only people we

can convince that castration is a good thing! We have supplemented our studies with specimens of testes obtained from volunteer prisoners.

Ageing in the human testis is a very gradual process. There is no period at which ageing processes are really precipitous. The testes from a man, 72 years old—the oldest individual we have—still look pretty good, and still have a certain amount of spermatogenesis going on. Beginning from about 45 years or so, and this is variable with individuals, spermatogenesis gradually slows down and together with this there is an increase in the number of cells which look like Sertoli cells. I call them Sertoli cells because their nuclear morphology and cytoplasmic inclusions, such as glycogen and lipid granules, are identical with those of Sertoli cells.

The oldest testes usually contain numerous Sertoli cells and sper-

Sertoli cells are numerous and not mixed with all the spermatogenic cells, one can see, even with the obsolete methods of the light or phase contrast microscope, a fairly distinct membrane between the individual cells.

one going towards the fibroblasts and one going towards the Leydig cells. I would like to hear Prof. Fawcett express his opinion on this matter.

Fawcett: Up to a year ago I was doubtful of the reported transition

believe that these cells are transitional forms between fibroblasts and mature Leydig cells. In the course of this study, therefore, I have become more receptive to the suggestion that has often been made in the literature to the effect that fibroblasts or fibroblast-like cells are transformed into Leydig cells during adult life.

Zuckerman: Would it be possible to arrange any experimental conditions in which the relative frequency of the transition from one to the other type of interstitial cell could be studied? I recall that when most cells which could possibly be spermatogonia disappear from the seminiferous tubule, and the cells of Sertoli become defined with considerable [illegible]

[illegible]

varies considerably—histologically—in different mammals. It would be interesting to examine different types of interstitial tissue with the electron microscope. I recall that in rodents, for example in mice and rats, that the cells are more fusiform or spindle-shaped with fewer epitheloid cells of the Leydig type than one sees in man, or, to cite another example, in the testes of deer. Thus there is a range to be [illegible]

[illegible]

cated the presence of steroid compounds in the cells. I wonder whether

the findings with the electron microscope have any bearing on the possible demonstration of ketosteroids in the rat's testis.

must include (1) the primary spermatocytes and spermatogonia, and (2) the Sertoli cells as sites of these substances. These elements give reactions identical with those found in the Leydig cells.

Fawcett: By electron microscopy, the Sertoli cells contain an abundance of neutral fat droplets but pigment is relatively uncommon. We have very rarely found either lipid or pigment in spermatogonia or spermatocytes.

Montagna: Judging only by the osmiophilia?

Fawcett: Judging by their appearance in electron micrographs of osmium fixed tissue. Osmiophilic pigment aggregations are easily distinguished from neutral fat in such preparations.

MITOCHONDRIAL CHANGES IN DIFFERENT PHYSIOLOGICAL STATES

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MITOCHONDRIA, defined by light microscopy as organelles stainable by selective stains and intravitally by Janus green, have been identified in sections examined with the electron microscope. They appear after osmic fixation as ovoid or elongated structures with an outer limiting membrane. This membrane is a double one. Its inner lamina is folded or extended into many inward projecting folds, each of which is composed of two closely apposed membranes. Enclosed by the limiting membrane and between the inner folds is a homogeneous, moderately dense matrix, in which there occurs occasional dark granules. The number and complexity of the internal laminar folds, the density of the matrix and the size and number of the granules all exhibit variations from tissue to tissue, but are reasonably similar from cell to cell in the same tissue.

We have examined mitochondria from several tissues in different physiological states, or after various experimental procedures. These organelles prove easily susceptible to alteration. They swell rapidly if fixation is delayed after the death of the animal. This post-mortem swelling can be prevented, however, by subjection to hypertonic solutions. Mitochondria behave, therefore, somewhat like osmometers.

After intravital exposure to several foreign or toxic reagents, mitochondria appear to concentrate the foreign substances and to become altered in the process. In rats, to whose drinking water silver nitrate had been added for long periods of time, deposits of dense granules occurred in basement membranes and in macrophages throughout the body and,

in addition, in occasional parenchymal cells. In the liver and pancreas, mitochondria, identifiable by their internal folds, were observed to contain silver granules. Further deposition of silver caused disruption of the internal folds and the formation of intramitochondrial vacuoles. Similar changes in mitochondrial appearance have been noted in our laboratory by Dr. Jules M. Weiss after intravital administration of neutral red. We have also seen occasional macrophages from normal animals in which granular aggregates were segregated within mitochondria.

The endodermal epithelium lining the yolk-sac cavity is a transient tissue in that it, like the rest of the placenta, lives its life only during the period of gestation. Moreover, in many animals, the yolk-sac is a flourishing organ early in pregnancy but becomes reduced in size and relative importance after the establishment of the chorioallantoic placenta. At midgestation in the guinea pig, mitochondria in the yolk-sac epithelium are normal in appearance and have well-defined internal membraneous folds. Near term, however, the mitochondria are swollen, their internal folds have vanished and many seem to have been converted into pigmented structures. In the mare's yolk-sac, which becomes reduced in size during the middle span of pregnancy, numerous cells become detached from the basement membrane. In these effete cells, swollen and disorganized mitochondria are the rule. These changes are not ascribable to poor or delayed fixation, since adjacent cells may contain mitochondria of normal size and structure.

The adrenal cortex can be regarded as a transient tissue in that moribund and dead cells are frequent in the inner, juxtamedullary zone, whereas mitoses are ordinarily encountered only in the outer zones. These and other cytological considerations lead to the assumption that cells in the outer portion of the zona fasciculata are in an active secretory phase; those in the inner fasciculata are becoming exhausted and those in the zona reticularis are dying. The electron microscopical appearance of these zones has recently been

studied in our laboratory by Dr. Jeffrey D. Lever. In the outer portion of the fasciculata, mitochondria are numerous and closely packed, they frequently have bizarre shapes and are closely associated with a system of membrane-lined vacuoles or tubules. Their internal structure differs from that exhibited elsewhere; the internal membranes here are tubular rather than laminar in configuration. The density of the mitochondrial matrix varies greatly, some mitochondria being excessively osmiophilic. In the zona reticularis the cells are smaller and the mitochondria are less closely packed; thus, it appears that with exhaustion of the cells, there is a loss of mitochondria.

Upon stimulation of the gland by exposure to cold or by administration of adrenocorticotrophic hormone, the mitochondria initially become less osmiophilic and their membranous structures become more sharply evident. More prolonged stimulation results in a restoration of the original osmiophilia. In prolonged stimulation, bizarre forms become more common and their structural alterations are more extreme. The limiting membranes are frequently re-duplicated and the internal folds become more complex. "Open" forms have been encountered in which the internum of the mitochondrion is continuous with the cytoplasm through pores in its limiting membranes.

These observations in various tissues and in different physiological states suggest that mitochondria are labile organelles, easily altered by changes in their physical environments. The experiments on vital staining show that the inclusion within cells of foreign agents is of considerable consequence to the integrity of their mitochondria. Ageing and moribund cells, exemplified by the yolk-sac and the zona reticularis of the adrenal gland, contain fewer and degenerating mitochondria. The experimental changes induced in mitochondria in the secretory zones of the adrenal gland offer hope, with further study, of understanding the manner in which they are formed and their relation to secretory processes.

DISCUSSION

Montagna: I would like to ask Prof. Dempsey whether or not he considers the mitochondria as fixed rather than labile structures. I find it difficult to reconcile the idea of mitochondria as rigid structures with the observations of Frederic and Chèvremont and with those of the Lewises. These investigators all observed living mitochondria undergo numerous vicissitudes around the nucleus.

two ago and confirmed Bensley's results. He then continued by refeeding the animal a normal diet, after the exhaustion of the mitochondria, and was able to show that the organelles regenerate with extraordinary rapidity. Within a matter of hours after administration of protein materials in the diet, the pancreatic cells again have their normal complement of mitochondria. In this kind of experiment it seems to me perfectly clear that they are labile structures. Also, in the adrenal cortex, there are many more mitochondria in the secretory zones than there are in those cells in the degenerating zones. If one permits the assumption that these are two different phases in the life-cycle of the same cell, then it would appear that mitochondria had been exhausted from the cell during its life-span.

Wislocki: This paper raises many questions about mitochondria; for

provide answers to many of these questions.

chondria but arise from some other structure in the cell.

Wislocki: That is a possibility.

mitochondria in electron micrographs of liver that appeared to be under

chondria divide or are formed anew from other components of the cytoplasm; but I have been disappointed in our own work to date, in that we seldom obtain pictures that provide clear-cut evidence for one or the other of these alternatives.

Dawes: I was puzzled by the use of the word "membrane"; could you explain that, Prof. Dempsey? It seemed rather like the story of Frankie and Johnny—that some of the membranes had no beginning and no end. Are they partitions?

Dempsey: Well, they are thought to be membranes in that mitochondria may be shown to be active osmotically. I quoted part of the evidence. It can also be shown that they can be swollen and shrunk [illegible]

some kind of terminology, and "membrane", in view of these two facts, seems to be tentatively reasonable.

Wislocki: We shall have to experiment with the effect of fixatives upon the preservation of the mitochondria in their most natural state. Besides artifacts introduced by fixation, mitochondria are subject to functional as well as age changes which must be recognized. It is difficult, with just the one or two fixatives now at our disposal, to say [illegible]

the matrix, one obtains an enhanced contrast of the essential skeleton of the mitochondria.

Dempsey: We prolonged fixation in Palade's buffered osmium solution

longer fixation so that the membranes stand out in sharper contrast.

MORPHOLOGICAL ASPECTS OF AGEING IN THE PLACENTA

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By modifying a previous definition devised by Flynn for vertebrate placentas, Mossman (1937) proposed that "the normal mammalian placenta is an apposition or fusion of the fetal membranes with the uterine mucosa for physiological exchange". Thus, the mammalian placenta is not a unified organ composed of homologous units, as the liver and kidney, but consists of several membranous structures which enter variously into relationships with one another in different groups of mammals. In any given animal, these membranous tissues collectively form the placenta, although some differentiate and function early in gestation, while others develop and assume placental functions later in pregnancy. Hence, the study of the ageing of the placenta is a matter of investigating not a single structural unit, but a variety of organs which may function either successively or concurrently. Thus, while some of the placental structures of a given animal are degenerating, others are growing and undergoing differentiation. One need only mention the successive or concurrent combination in mammals of a bilaminar yolk-sac placenta, a choriovitelline placenta, an inverted yolk-sac placenta (complete or incomplete), a chorionic placenta and a chorioallantoic placenta. The combinations and sequences of these structures in various placental types are catalogued and described in masterly papers by Mossman (1937) and Amoroso (1952).

Many of these placental structures are provisional or transient and serve only temporarily to support the growing conceptus. Thus, the bilaminar omphalopleure and choriovitelline membranes may be eventually completely resorbed

(tree-shrew, vespertilionid bats, lemurs (Loris, Galago), horse, coney (Hyrax)). Following complete inversion of the yolk sac (lagomorphs, many rodents), its parietal wall, as well as the chorion and the decidua capsularis associated with it, degenerate completely. In the pig's placenta the unvascularized ends of the chorionic sac wither. In the human placenta, the chorion laeve and decidua capsularis are provisional in nature and disappear completely by the fourth month of gestation. The majority of these transient foetal structures are functional and subserve physiological exchange until regression sets in. Moreover, in most mammals, the uterine endometrium undergoes various degrees of destruction and resorption during the course of gestation as a result of being invaded by the trophoblast of the chorioallantoic placenta. The degenerating endometrium forms an important source of nutrient material (histotrophe) for the growing placenta and conceptus. From all of this it is evident that considerable portions of the placenta undergo ageing and ultimate death long before pregnancy is over. These losses are compensated by other parts of the foetal membranes which differentiate into the definitive placental structures which mediate physiological exchange until the end of gestation.

The definitive parts of the placental membranes of Eutherian mammals, which include a chorioallantoic placenta and sometimes a yolk-sac placenta, undergo progressive histological and cytological changes which have been the subject of numerous investigations (cf. Amoroso, 1952). Histochemical changes in the placenta have also been investigated in a variety of mammals including the sow (Wislocki and Dempsey, 1946a), sheep (Wimsatt, 1950, 1951), cat (Wislocki and Dempsey, 1946b), shrews (Wislocki and Wimsatt, 1947), bat (Wimsatt, 1948, 1949), rat (Wislocki and Padykula, 1953) and man.

The human placenta of the second and third months of

sey, 1948; Wislocki, 1955). It has been compared at the two periods with respect to the localization and amount of nucleoproteins, enzymes, carbohydrates and lipids which it contains. Electron microscopy has also revealed significant cytological differences between the placenta of the first trimester and at full term (Wislocki and Dempsey, 1955a).

The cytological and cytochemical changes in the human placenta involve both the nuclei and cytoplasm of the trophoblast (Wislocki, 1955). Mitotic division decreases and various cytoplasmic structures and substances diminish in size and amount. The lipid droplets and mitochondria of the trophoblastic syncytium decrease both in size and number. The brush border of the syncytium seen with the daylight microscope, and the equivalent microvilli observed with the electron microscope, diminish in length. Glycogen gradually disappears from the foetal placenta. Cytoplasmic ribonucleoprotein, seen in the syncytium as cytoplasmic basophilia in the daylight microscope and as ergastoplasmic vesicles in the electron microscope, decreases. On the other hand, acid and alkaline phosphatases increase. The stroma of the villi becomes less cellular and more fibrous.

Biochemical methods afford another means of determining the amount and direction of placental changes during gestation. Thus, in the human placenta, Villee (1955) reports a gradual decline in the amount of placental glycogen, in the degree of anaerobic glycolysis, and in the rate of oxygen consumption. With respect to the decline in glycogen, the histochemical and biochemical observations are in agreement.

A recent study of the enzymatic reactions of the rat's placenta from the 18th day of gestation until full term reveals a number of changes (Padykula, 1955, 1956). Adenosine triphosphatase and glycerophosphatase (pH 9·4), acid phosphatase, esterase (Pearse's method) and succinic dehydrogenase (Seligman and Rutenburg's method) were investigated in frozen, cryostat sections. Homogenized visceral yolk sac was also assayed biochemically for succinic dehydrogenase, adenosine triphosphatase and glycerophosphatase activities.

The several enzymatic reactions were lowest at 13 days in all parts of the rat's placenta. In the visceral endoderm of the yolk sac, alkaline glycerophosphatase reached a maximum at 17 days and declined to almost none by 21 days. Adenosine triphosphatase rose to a maximum at 19 days but then declined somewhat by the end of gestation. Acid phosphatase activity rose steadily until the end of pregnancy. Esterase activity reached a peak at 15 days and diminished slowly thereafter. Succinic dehydrogenase activity was maximal on the 16th day and then declined rapidly. Assays of homogenates of the yolk sac confirmed the histological findings for adenosine triphosphatase, alkaline glycerophosphatase and succinic dehydrogenase. In the chorioallantoic placenta, adenosine triphosphatase activity increased markedly in the spongy zone between the 17th and 19th days, as did also acid phosphatase between the 15th and 19th days.

Various cytological and cytochemical changes have been reported in the placenta of the rat (Wislocki and Padykula, 1953). For example, glycogen begins to accumulate in the visceral endoderm of the yolk sac on the 15th day, reaches a peak on the 18th day, and declines thereafter. A mucopolysaccharide, demonstrable by the periodic acid-Schiff reagents, increases from the 10th to 16th days and then declines (18th day), but increases once more by the 21st day. In the trophoblast of the placental labyrinth, where glycogen is never abundant, it increases up to the 18th day and subsequently diminishes. In the spongy zone and the subplacental myometrium, glycogen is extremely abundant up to the 18th day but then declines rapidly.

The decrease of some substances in the placenta may be related to the assumption of functions by various foetal organs, especially the liver. From study of the rabbit's placenta, Claude Bernard (1895) advanced the idea that the placenta serves as a deputy for the synthesis and storage of glycogen until the foetal liver begins to function. In confirmation of this, glycogen first appears in the liver of the rat on the 19th day of gestation when it has begun to disappear from the

placenta (Padykula, 1955). Similarly, it has been suggested (Wislocki, Dempsey and Fawcett, 1948) that the intense cytoplasmic basophilia of the early human placenta, which is attributable to ribonucleoprotein, represents a provision for the synthesis of proteins necessary for foetal growth until such time as this function is taken over by the foetal liver.

A progressive increase in permeability of the placental barrier has been reported by various investigators. In rabbits, placental permeability to antibodies (Rudolfo, 1934), phenolsulphonphthalein (Lell, Liber and Snyder, 1932), neoarsphenamine (Snyder, 1943) and radioactive sodium (Flexner and Pohl, 1941*a*) increases during the course of gestation. Similarly, in the rat there is a gradual increase in permeability to insulin (Corey, 1932) and radioactive sodium (Flexner and Pohl, 1941*b*). Moreover, Flexner and his associates have observed an increase in the rate of transfer of heavy water and sodium across the placental barrier in other mammals (sow, goat, cat, man). These increases in the rate of placental exchange have been ascribed to a progressive reduction in the number of layers of the placenta accompanied by a diminution in width of the barrier, a combination of changes which has generally been regarded as favouring a progressive increase in placental "efficiency".

Aside from these gradual morphological and physiological changes, the question arises as to whether, beginning some time late in gestation, the definitive placental structures undergo regressive, terminal changes which have an unfavourable effect on placental exchange, limit the length of gestation, or influence the time of parturition. Such changes, if present, would comply with Lansing's (1952) definition of ageing as a process of unfavourable progressive change which becomes apparent after maturity, is inversely related to growth and involves a decrease in efficiency of the mechanism for reconstruction.

There are relatively few substantial morphological grounds upon which to evaluate to what degree the definitive placental structures undergo unfavourable terminal changes which

depress physiological transfer or influence the onset of labour. In the human placenta, in which more attention has been devoted to this problem than in animals, unfavourable, terminal morphological changes have been described. These include the accumulation of fibrin, "fibrinoid" and calcium (Hertig, 1946), the "hyalinization" and loss of the syncytium covering a variable number of the chorionic villi (Tenney, 1936), and haemorrhagic infarcts involving the complete sclerosis of placental villi (Thomsen, 1955). However, to what degree and how these changes actually limit or decrease the overall efficiency or individual functions of the human placenta is mainly a matter of conjecture.

With respect to animals, the epithelium of the visceral layer of the yolk-sac placenta of the guinea pig, investigated with the electron microscope (Dempsey, 1953), reveals that the mitochondria at full term are swollen and some are degenerating. Furthermore, the cell cytoplasm is more granular than earlier in gestation and some of the ergastoplasmic structures are swollen. In the rat, however, which has a much shorter gestation period than the guinea pig, similar degenerative age changes have not been observed (Wislocki and Dempsey, 1955*b*).

These differences between the ageing of the yolk sacs of the guinea pig and rat raise the interesting question as to whether the definitive placentas of animals with long gestation periods show more terminal age changes than those with short periods. The shortest gestation periods for Eutherian mammals are 16 days for the golden hamster and 17 to 19 days for the short-tailed shrew, whereas the longest periods are over 600 days for the elephant and between 420 and 500 days for the giraffe and rhinoceros. Perhaps the most rewarding comparison of the degree of terminal ageing with the length of gestation could be carried out in rodents. There, differing from most orders, the length of gestation varies greatly in different species, whereas the definitive chorioallantoic and yolk-sac placentas are relatively similar in their basic structure. One might profitably compare the terminal placental histology of

species of rodents which have the shortest gestation periods (hamsters, mice, rats) with that of members which have periods six to seven times as long (porcupines, beavers).

A sharp decline in the last decile of pregnancy has been observed by Flexner and Gellhorn (1942) in the rate of transfer of radioactive sodium across the placentas of various mammals (rodents, rabbit, cat, goat), excepting the sow in which there is no diminution. A similar decline in sodium transfer has also been reported in the human (Flexner *et al.*, 1948), which Flexner (1955) attributes to the deposition of "fibrin over the villi" and to "thromboses". This unfavourable terminal change in placental transfer of sodium would seem to be valid, although there are no morphological expressions of terminal senescence, presently known in the animals cited, with which to correlate the results.

Of the various cytomorphic and biochemical changes which occur in pregnancy, it is difficult to say specifically which are progressive and which regressive. The terminal change in sodium transfer found by Flexner in various mammals and the changes in the mitochondria recorded by Dempsey in the guinea pig's ageing yolk sac might well be regarded as regressive. The decline in adenosine triphosphatase and of succinic dehydrogenase noted in the rat's yolk sac by Padykula might also be thought of as signalizing regression, especially since adenosine triphosphatase plays a rôle in general energy release. But for the majority of changes no definite clues exist as to whether they exert favourable or unfavourable influences upon placental exchange.

Premature and pathological age changes have been described in the human placenta, but they differ only in degree from the normal terminal regressive changes alluded to. As a consequence of the difficulty of measuring or assaying quantitatively the differences between normal and pathological placental ageing, it has proven impractical to distinguish ordinary terminal changes from premature and pathological ones.

The toxaemias of pregnancy and eclampsia have been

variously ascribed to a variety of placental lesions. Premature and excessive degeneration of the syncytial trophoblast has been held responsible for these conditions (Tenney, 1936; Tenney and Parker, 1940), and, since the syncytium is regarded as the site of formation of placental ketosteroids (Wislocki and Bennett, 1943; Wislocki, 1955), the observed changes in ketosteroid metabolism in the toxaemias have been ascribed to syncytial degeneration (Smith and Smith, 1948; Sommerville, 1950). Bartholomew and his associates (1932, 1934, 1936) have attributed the toxaemias to spastic constrictions of the foetal blood vessels of the chorionic villi, resulting in the formation of red infarcts with necrosis of the villi in the infarcted areas. Thomsen (1955) agrees with him, except that he regards the placental lesions as secondary rather than as the primary precipitating cause of eclampsia. Many other speculative explanations of the toxaemias of pregnancy have been advanced (cf. Dieckmann, 1952). Schneider (1950), for example, attributes these conditions to the toxic effects of thromboplastin liberated from pathological haematomas which frequently occur between the decidua and the basal placental plate late in pregnancy. Page (1953) offers a theory involving an unidentified vaso-toxic substance produced in the placenta, combined with a high sodium intake and the presence of large quantities of placental steroids.

In two cases of pre-eclampsia, Flexner and his associates found a marked reduction in placental permeability to radioactive sodium. In toxaemia the ability of the placenta to concentrate amino acids is also decreased (Crumpler, Dent and Lindan, 1950; Villee, 1955).

One avenue of investigation of terminal placental ageing remains to be mentioned, namely, the postmature retention of foetuses *in utero* induced by inhibition of parturition. This was first accomplished experimentally by producing a fresh set of corpora lutea in the rabbit on the 28th day of pregnancy (Snyder, 1934), and later by others as the result of injecting progesterone. Following this procedure, the foetuses remain alive and continue to grow for 4 to 6 days beyond the normal

time of parturition, but eventually die when they become too large to be delivered. It would be interesting to investigate both the cytology and the functional capacities of the placentas of such postmature rabbit foetuses. In connection with postmaturity, Masters (1952) cites three cases in women of intra-abdominal pregnancy which were terminated by Caesarean section at or near term, and the placenta allowed to remain in the abdominal cavity; for three weeks thereafter, active chorionic gonadotrophin and sodium pregnanediol excretion was present.

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DISCUSSION

Hamilton: I was very interested to hear what Prof. Wislocki had to say on the placenta. Anything he has to say concerning the placenta is always received with very great interest.

[illegible]

In the early stages, the endothelium, of course, is quite thin but as gestation proceeds it becomes thickened in many places and indeed many-layered. Why this occurs is not very easily answered but it is a

illi in the early advances the

been described by the pathologists.

We also found that syncytium invades the mouths of endometrial vessels, both veins and arteries, that are opening into the intervillous spaces, or what we would rather regard as labyrinthine spaces than purely intervillous spaces.

Amoroso: In your allusion to the endometrium, are you making a distinction between the maternal part of the placenta and other parts of the endometrium?

Hamilton: I am.

Harrison: One of the points I would like to raise is the question of increase in surface area of the placenta as it ages or as it grows. Prof. Hamilton and I have been working on a series of Fallow Deer which have been obtained throughout pregnancy and we have specimens covering extensively the period of gestation in this animal. We find

to make on that in other mammals.

Wislocki: That question was extensively discussed at the First Conference on Gestation of the Josiah Macy Jr. Foundation, 1955. It was stressed that unit weights do not form a valid basis for comparison of the relative rates of placental exchange in different mammals. The respective surface areas through which metabolic exchange occurs, involving either the effective surface of the placental membranes (trophoblast, vitelline membrane) or the surface area of the placental capillaries, would seem to be the most important factors.

Dawes: In the whole animal perhaps what matters is the total amount of exchange. If you define "efficiency" in terms of surface area, you

CHRONOLOGICAL CHANGES IN PLACENTAL FUNCTION

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Placental functions can be examined in two aspects, permeability effects and endocrinal functions. The first covers transmission of oxygen and nutrients from the mother and of carbon dioxide and waste products from the foetus. The second covers the well-recognized synthesis of reproductive endocrines which affect the mother and possibly the foetus. It also includes production of materials of metabolic importance which may be demonstrable in placental cells before disappearing and are to some extent the basis of Claude Bernard's dictum that the placenta is the liver of the foetus.

Unfortunately, while many different aspects of placental function have been studied, there are only a limited number of investigations effected at different conceptional ages and it is only possible to mention a few in the time at one's disposal.

Gellhorn and Flexner (1942) studied the passage of radioactive sodium across the placenta of the rabbit and other species (Fig. 1). These show increasing permeability until within 10 per cent of full term but a fall off in the last 10 per cent of intra-uterine life. Whether this last 10 per cent is the effect of postmaturity is a point of debate, as is also the standard of reference.

Rodolfo (1934) found that the amount of antibodies passing during gestation increased, but the rate of increase was progressively diminished. There was no falling off. Barron (1951) has investigated the oxygen passage across the placenta in the sheep. There is evidence of different degrees of transfer at different placental ages and increasing oxygen tension in the umbilical vein blood in the last few days.

My colleague, Dr. Wilfred Widdas, injected into pregnant

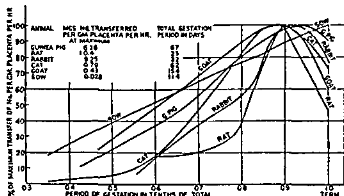
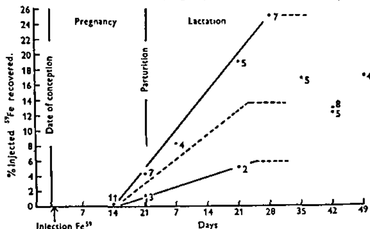


FIG. 1. Transfer of sodium across unit weight of placenta in unit time in different species. (After Gellhorn and Flexner, 1942.)

rats an amount of ^{59}Fe adequate to produce a constant specific activity in the mother which may possibly also have been the maximum saturation. There was a rise in the total iron in the litters and an increasing percentage of labelled Fe was recovered from the litters (Fig. 2). But when the iron/foetal



weight was calculated it was seen that a relative anaemia was produced (Fig. 3). This, however, was not due either to shortage of iron or inefficiency of the placenta but to the foetal growth being disproportionate.

At the risk of overlapping with Professor Wislocki's approach, one would draw attention to the weights of the human placenta given in Adair and Thelander's classical paper in 1925. In no case is there any suggestion that the placenta loses weight as the foetus grows (Fig. 4). In the goat, however, Elliott, Hall and Huggett (1934) found evidence of

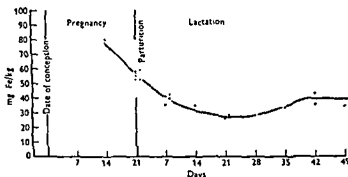


Fig. 3. Ratio Iron/Weight in foetal and newborn rats. (Huggett and Widdas, 1949.)

a slowing up or flattening out of the placental weight curve. On the other hand, umbilical blood flow through the placenta in the foetal sheep has been shown to rise rapidly as term approaches (Fig. 5) (Cooper, Greenfield and Huggett, 1949).

When considering the production of materials by the placenta one should draw attention to the production of carbohydrates within it. Mammalian allantoic placentas can be divided into two classes, those whose foetal blood has one sugar, glucose, and those whose foetal blood has two, glucose and fructose, and in these, fructose is always in excess. These latter are all Ungulata or Cetacea. Glucose can flow back to the mother if the gradient is reversed experimentally but fructose never does. This fructose is synthesized by the

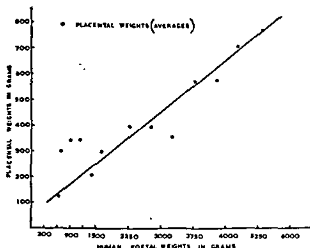


FIG. 4. Relation between placental and foetal weights in the human. (After Adair and Thelander, 1925.)

placenta, a function not possessed by the foetus. This synthesis appears to be a steadily increasing function, as shown by Table I. All the other mammals have no foetal fructose and always appear to have much glycogen at some stage in the

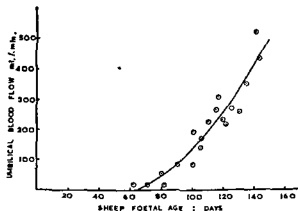


FIG. 5. Umbilical blood flow in the foetal sheep at different ages. (After *Cooper, Greenfield, and Huggett*, 1949.)

Table I

Total Fructose Secretion by the Placenta

Foetal age (days)	*Blood fructose (mg.%)*	*Blood volume (ml.)*	*Fructose in foetal blood (mg.)*	*Total fructose space (ml.)*	*Total fructose in fructose space (mg.)*	*Corrected by Hitchcock (1949) (mg.)*
60	150	15	22·5	130	195	225
80	125	50	62·5	200	250	312
100	100	110	110	300	300	375
120	75	250	187	430	325	406
140	50	500	250	1800	900	1125

placenta. All placentas have deposits not only of glycogen but several other materials which all have dates of maximal deposition differing with the material and the species (Table II). It would appear that as the placenta ages it takes on

Table II

Endoplacental Composition in the Rodent
Dates of Maximum Concentrations
(Full-term: Rabbits, 30 days; Rats, 21 days)

Substance	*Day of maximum concentration*	*mg.%*	*Reference*
Rabbit			
Glycogen (foetal)	14	1·54 g./100 ml.	Lochhead and Cramer (1908)
Glycogen (maternal)	21	5·57 g /100 ml.	Lochhead and Cramer (1908)
Rat			
Glycogen (whole placenta)	16	830	Huggett (unpublished)
Rabbit			
Phospholipin	30	1500	Boyd (1935)
Free cholestrol	30	275	,, ,,
Cholestrol ester	21	400	,, ,,
Neutral fat	17	970	,, ,,

different functions, not only as a whole but in different parts of the placenta and in different species.

The peak of placental glycogen in the rat is at the 16th day, after which it decreases. Some years ago my former colleague, Professor J. J. Pritchard, and I investigated the effect on the placenta of experimental foetal death in the rat (Huggett and Pritchard, 1945; Pritchard and Huggett, 1947). Oestrogens caused decidual necrosis and foetal death but

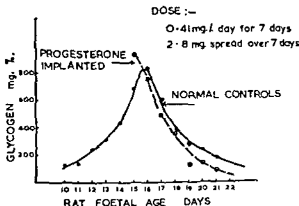


FIG. 6. Daily averages of placental glycogen in the last third of pregnancy in normal pregnant rats and rats implanted with progesterone during the previous 7 days. Average absorption 0·28 per day.

progesterone had no such effect; rather the foetus thrived. An extension of these experiments has demonstrated that the implantation of progesterone during a period of 7 days in the pregnant rat with an average absorption of 0·41 mg. per day per rat produces an accelerated decrease of the glycogen in the period of placental glycogen decrease (Fig. 6). It also causes a decrease of placental weight accompanied by an increase of foetal weight (Figs. 7 and 8).

In summary, therefore, the placenta changes composition not in a regular sequence to old age but spasmodically, suggesting that different functions are exercised at different

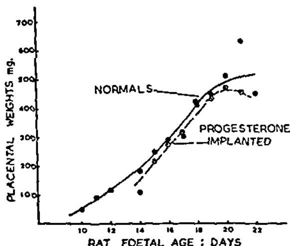


Fig. 7. Daily averages of placental weights in these same experiments.

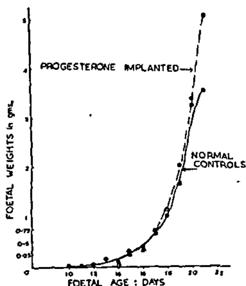


Fig. 8. Daily averages of foetal weights in these experiments.

ages, and there is evidence that its "efficiency", certainly for some functions when considered in relation to foetal growth, is increased in its old age; in fact, it might be said to be abruptly expelled in the prime of life.

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DISCUSSION

Amoroso: It is evident that Professor Huggett does not feel inclined to accept Lansing's definition of the ageing process. As I understand it from what Professor Wislocki has said, Lansing considered the absence of the powers of reconstruction in an ageing tissue to be an important criterion of sensescence.

Dawes: I would like to say how much I enjoyed Prof. Huggett's exposition of the [illegible] of the placenta. I think that in any dis-

placenta rose progressively during pregnancy. Now, blood pressure also rises during the latter half of pregnancy, and it would seem that this rise in blood pressure is in fact due to the development within the foetus of vascular reflexes and the sympathetic nervous system. I wonder, therefore, whether we should not concentrate attention, in future work, on trying to find out what happens to the vascular resistance on the foetal side of the placenta. So far as the little evidence available suggests, we may find that the vascular resistance is the same throughout the latter part of pregnancy, and the increase in umbilical blood flow may be due to an increased cardiac output and to changes in the foetus rather than the placenta.

In any discussion of the transfer of material across the placenta, there are obviously three variables we have to take into account, first, the foetal placental flow, secondly the diffusion coefficient of the placental tissues, and thirdly uterine blood flow. Now, as to uterine blood flow there is practically no data available. Barcroft made some observations in rabbits but the numbers of rabbits were rather small. I know that there is great technical difficulty in measuring maternal

efficiency has been firmly established, but in order to investigate it, uterine flow must be measured.

Amoroso: Were you comparing the sheep with the rabbit in respect of maternal flow?

Dawes: No, the only published work is on the rabbit.

Amoroso: I raised that point because we might get into difficulties if we try to relate results derived from the rabbit to sheep and *vice versa*. I think that point was raised, or at least was hinted at by Professor Wislocki when he referred to the maturation of the foetus. The sheep foetus is, as we all know, a very much more mature organism at term

dominal pregnancies in castrated rabbits by opening the uterus at the level of a nidation, and castrating the female on the same day. The

intra-uterine foetuses died quickly after castration, the abdominal ones survived and grew. Progesterone is necessary only for the intra-uterine development.

Castration in the pregnant rat does not always stop development of all foetuses. Some may survive, but they are often deformed by uterine pressure. In a series of personal experiments, the weight of such foetuses found alive on day 21 was about 4 4 g., with controls of the same strain of 4·0 g. Despite the uterine impairment, foetal growth was not very much reduced by the absence of the corpora lutea.

Amoroso: Of course, you make the assumption that there is no extra gonadal source of progesterone there.

Jost: Yes, but in the rabbit and in rats there is not too much progesterone present; at least it has never been detected.

Amoroso: There is enough, nevertheless, to maintain abdominal pregnancies in ovariectomized rabbits. Moreover, I believe that both Professor Courrier and Professor Klein would dispute your point.

Huggett: May I just comment on these two points; first, I entirely agree, I do not think progesterone is necessary for the growth of the [illegible]

weights increase. So this raises the question whether the placenta [illegible]

function?

T.-Duplessis: The foetal-maternal exchange. Since these foetuses are still alive 3 or 4 days after term and are bigger than normal we may assume that the placenta does function correctly.

Amoroso: A considerable amount of work has been carried out on the prolongation of pregnancy in these animals by Hill and Parkes, and by Professor Wislocki and his associates which antedates the work of Sir Joseph Barcroft.

Huggett: Yes, and they showed that if the foetus does go on too long, then it asphyxiates itself.

Amoroso: For my guidance, might I ask Professor Huggett what [illegible]

the chorioallantoic membrane, so what was weighed were the caruncles and placentomes and not the placenta.

There are two points; first, Dr. Fahmy and I gave a paper at the Federation in Atlantic City last year (1954) in which we showed that

Secondly, as the placenta gets thinner, then it is more efficient; diffusion goes on. As far as one can see from the work being done, the physio-chemical diffusion as such plays practically no part at all. It is only between thinning and diffusion so that it is almost a facilitated, a

described and are figured in *Brit. Med. Bull.* (1955) vol. 11.

BIOCHEMICAL EVIDENCE OF AGEING IN THE PLACENTA

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THE concept that the placenta undergoes regressive changes in the latter months of gestation and that pregnancy is terminated when the placenta can no longer provide an adequate supply of nutrients to the child was originally stated by Hippocrates (Reynolds, 1949). Careful histological and histochemical observations (Wislocki and Bennett, 1943; Wislocki, Dempsey and Fawcett, 1948) have provided evidence of thickening and hyalinization of the walls of the blood vessels and of the presence of thromboses in the last two or three months of gestation. Decreases in the rates of oxygen consumption and of anaerobic glycolysis as gestation proceeds have been reported (Loeser, 1932; Wang and Hellman, 1941; Page, 1948; Hellman, Harris and Andrews, 1950). The present paper describes the results of investigations of the intermediary metabolism of slices from placentas ranging in age from six weeks to term. The metabolic activities of the placenta decrease as gestation proceeds, but the decreases are not general and uniform. The decreases in activity are gradual, without sharp change near term, and the placenta at term is still quite active in many respects.

Term placentas were obtained directly from the delivery room and were used within five minutes of delivery. Earlier placentas were obtained at Caesarean sections performed for delivery or for therapeutic interruption of pregnancy and were used within five minutes of their removal from the patient's body. Placental slices, about 0·5 mm. thick and weighing about 200 mg., were cut with a Stadie-Riggs microtome or with fine scissors. Care was taken in preparing the slices to avoid areas of necrosis. The slices were incubated

in Warburg respirometer vessels at 37° in an oxygen atmosphere. The composition of the incubation medium, expressed in millimoles per litre, was K^+ 50, Na^+ 80, Mg^{++} 10, phosphate 40, Cl^- 100, pyruvate 10 and glucose 11·1. In alternate vessels, either the glucose or the pyruvate was labelled with ^{14}C. The initial pH of the medium was 6·8 and the pH after incubation was 6·8 ± 0·1 (determined by glass electrode). Other slices of the placentas were used for determination of initial glycogen content and of wet weight: dry weight ratios.

After a two hour incubation, the slices were removed, weighed, and analysed for glycogen. Aliquots of the incubation medium were analysed for glucose (Nelson, 1944), pyruvate (Friedemann and Haugen, 1943), and lactate (Barker and Summerson, 1941). The respiratory CO_2 was recovered from the alkali in the centre well and converted to $BaCO_3$, then its ^{14}C content was measured with a windowless, proportional flow counter (Robinson, 1950). Glucose was isolated from other aliquots of the incubation medium, after the addition of 10 mg. of carrier glucose, as the glucose phenylosazone. This was washed, recrystallized, washed again, and its radioactivity was measured with the flow counter. Pyruvate was isolated from the other aliquots, after the addition of 10 mg. of carrier pyruvate, as the 2:4-dinitrophenylhyrazone. This was washed seven times with alcohol and water, then its radioactivity was measured with the proportional flow counter. Aliquots of the glucose obtained from the tissue glycogen were converted to glucosazone for radioactivity determinations.

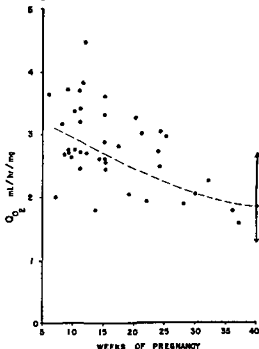
From these measurements one can estimate the net amount of glucose utilized or produced, the net amount of pyruvate utilized, the net amount of lactate produced, the amounts of glucose and pyruvate carbons metabolized to CO_2, the net amount of glycogen produced or utilized, the amounts of glucose and glycogen made from pyruvate, and the amounts of glycogen and pyruvate made from glucose.

Preliminary experiments showed that there was no detectable difference in the metabolism of slices from the chorionic

and decidual surfaces of the placenta and that slices cleaned of blood by exhaustive washing with cold saline and those cleaned by blotting on filter paper showed similar rates of metabolism. Slices prepared from placentas which had been standing 60 minutes at room temperature showed metabolic rates 20 per cent less than those cut immediately.

Oxygen Consumption

A gradual decrease in the rate of oxygen consumption of the placenta as gestation proceeds has been reported previously (*loc. cit.*). The present series includes thirty term placentas



[illegible] placenta.

and thirty-three earlier placentas, ranging in age from 6 to 37 weeks. The decrease in oxygen consumption with age is linear until a gestational age of about 30 weeks, after which the Q_{O_2} (microlitres of oxygen utilized per mg. of dry weight of tissue per hour) is relatively constant until term (Fig. 1). The values for oxygen consumption reported here are slightly lower than those reported by Hellman, Harris and Andrews (1950), probably because of the differences in the method of determining the dry weight of the tissue. In their experiments the tissue was removed from the medium at the end of the incubation period, dried and weighed. We have found that the wet weight of the tissue recovered at the end of the experiment is only 60 to 70 per cent of that put in originally. To avoid this source of error, the dry weight : wet weight ratio for each placenta was determined from adjacent slices. The placental slices recovered from the incubation medium at the end of the experiment were weighed and their glycogen content was measured. The ratio of dry to wet weight of the placenta almost doubles during gestation (Fig. 2).

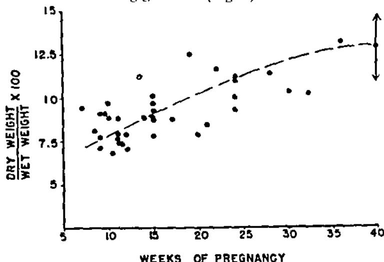
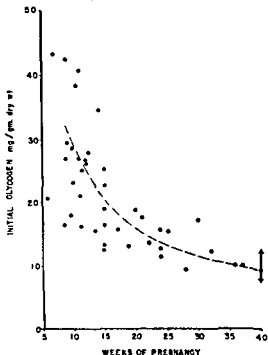


FIG. 2. The ratio of dry to wet weight of the placenta as a function of placental age. The arrow at 40 weeks indicates the range of the values obtained for term placenta.

Glycogen Content and Metabolism

The glycogen content of the placenta was estimated by the method of Walaas and Walaas (1950). The glycogen was isolated and purified, hydrolysed to glucose, and the glucose



[illegible]

was measured by the method of Nelson (1944). There is a [illegible]enta with [illegible] glycogen [illegible] 12 to 15 weeks of pregnancy.

From the measurements of glycogen content before and

after incubation, the net change in glycogen content was calculated and expressed as micromoles of glucose units per g. of wet tissue per hour. The placenta early in gestation has a marked ability to synthesize glycogen *in vitro* (Fig. 4). This ability begins to decrease after 10 to 12 weeks of pregnancy and decreases steadily to term. The average rate of glycogen utilization of the thirty term placentas was −1·92 micromoles per g. per hour, and only one of the thirty showed a

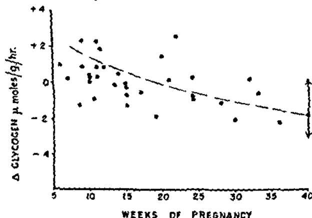
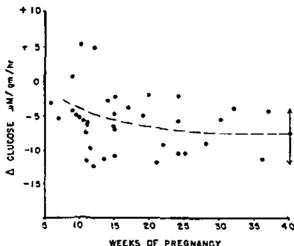


FIG. 4. The metabolism of glycogen by the placenta as a function of age. Positive values indicate net glycogen synthesis; negative values indicate net glycogen utilization. The values are expressed as μmoles of glucose units per g. of wet tissue per hour. The arrow at 40 weeks indicates the range of values for term placenta.

net production of glycogen (+0·22 micromoles per g. per hour). Corroborative evidence was obtained from the radioactivity measurements of the glucosazones derived from the tissue glycogen after incubation. These showed that term placentas have a just barely detectable ability to incorporate [^{14}C]glucose into glycogen *in vitro*. In contrast, 8 to 14 week placentas have a marked ability to incorporate ^{14}C from both glucose and pyruvate into glycogen *in vitro*. This ability decreases with gestation and was absent from two of the three placentas aged 22 to 24 weeks.

The Metabolism of Glucose

The ability of the placenta to utilize or produce glucose was calculated from the difference in glucose content of the incubation medium before and after incubation. Some of the earliest placentas tested showed a net production of glucose during the incubation (Fig. 5). From 15 weeks until term, however,



[illegible]

tissue per hour. The arrow at 40 weeks indicates the range of values for term placenta.

the average net rate of glucose utilization proved to be about −7 micromoles of glucose per g. wet tissue per hour. From the difference in the specific activity (c.p.m. per millimole glucose) of the glucose in the incubation medium before and after incubation, one can estimate the amount of glucose produced by the placenta and secreted into the medium and the true amount of glucose utilized. These calculations showed that the placenta at 10 weeks has a glucose production of about +12 micromoles per g. per hour and a true glucose utilization of about −17 micromoles per g. per hour. The net

glucose utilization is, therefore, -5 micromoles per g. per hour. At term, the true glucose utilization is $-7 \cdot 5$ micromoles per g. per hour, glucose production is zero, and the net glucose utilization is also $-7 \cdot 5$ micromoles per g. per hour. Hence, although the average net utilization of glucose is fairly constant from 15 weeks to term, this is in fact due to concomitant decreases in both the production and utilization of glucose.

The production of glucose by the liver has been shown to be mediated by the enzyme glucose-6-phosphatase (de Duve, Berthet, Hers and Dupret, 1949; Swanson, 1950), a reaction in which glucose-6-phosphate is converted to glucose plus inorganic phosphate. The glucose-6-phosphate is derived from glycogen via phosphorylase and glucose-1-phosphate, or by gluconeogenesis from other carbon compounds by a reversal of glycolysis. The human foetal liver gains the ability to secrete glucose only after 12 to 16 weeks of development (Villee, 1953*a*). Before that time it is unable to regulate the concentration of glucose in the foetal blood stream.

The placenta does have the ability to secrete glucose early in pregnancy but loses this ability as gestation proceeds. The placenta thus could regulate the concentration of glucose in foetal blood early in development but not later.

The ability of a tissue incubated *in vitro* to secrete glucose into the incubation medium may be tested in three ways. First, if glucose production exceeds glucose utilization, direct chemical analysis of the medium will reveal the net increase in the amount of glucose present. Second, whether or not there is a net increase in glucose concentration, glucose production can be detected by using ^{14}C-labelled glucose in the incubation medium and measuring the specific activities of glucose isolated from the medium before and after incubation. If the tissue produces glucose, the unlabelled glucose molecules produced will dilute the labelled molecules of glucose present in the medium and the specific activity will therefore decrease as incubation proceeds. Third, the tissue may be incubated in the presence of ^{14}C-labelled pyruvate, or in the

presence of any substance which is readily metabolized to glucose-6-phosphate. Glucose is isolated from the medium at the end of the incubation period, and its radioactivity is measured. In this way the production of glucose can be demonstrated directly and its rate of formation from the given precursor can be estimated. By all three methods it was shown that the placenta can secrete glucose early in pregnancy. However, it gradually loses this ability, presumably by a loss of the enzyme glucose-6-phosphatase (Table I).

Table I

THE METABOLISM OF GLUCOSE BY PLACENTAL SLICES

Age (weeks)	*No. of exp.*	*μmoles/g. tissue hour*		
		Glucose utilized	*Glucose produced*	*Glucose produced from pyruvate*
8–10	4	−17·7±0·53*	+11·9±1·5	+0·27±0·01
11–12	6	−19·3±1·1	+ 9·5±1·5	+0·19±0·02
13–15	5	−18·6±3·6	+ 9 9±2·8	+0·16±0·05
19–23	5	−22·3±1·9	+14·3±2·1	0
28	2	−15·9	+11·7	0
32	1	−12·8	+ 9·0	0
36	2	−11·2	0	0
40	12	− 6·6±0·68	0	0

* mean ± standard error of the mean.

These experiments demonstrate that the placenta early in gestation has the biochemical mechanism necessary for the storage of glycogen and the secretion of glucose. It could function to regulate the glucose content of the foetal blood stream. Claude Bernard demonstrated the presence of glycogen in the sheep placenta and in 1858 made the suggestion that the placenta may act as an "accessory liver".

The Metabolism of Pyruvic and Lactic Acids

The net utilization of pyruvate, calculated simply from the difference in the content of pyruvate in the medium before and after incubation, by placentas at successive stages of

gestation is given in Fig. 6. There is a considerable amount of variation between placentas of the same age. A slight decrease in the net utilization is evident after 10 weeks of development. The amount of pyruvate produced during the incubation period can be calculated from the dilution of the radio-pyruvate in the medium by unlabelled pyruvate produced by the cells. The true utilization, i.e., the net

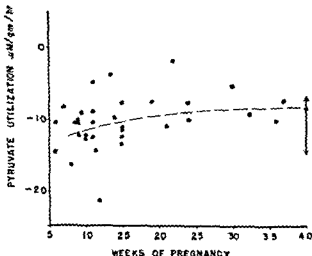


FIG. 6. The utilization of pyruvate by the placenta as a function of age. The values are expressed as μmoles per g. of wet tissue per hour. The arrow at 40 weeks indicates the range of values for term placenta.

utilization corrected for the amount produced, can then be estimated. Both production and true utilization of pyruvate increase from 7 weeks to 24 weeks and then decrease at term (Table II). The net utilization based on these calculations (column 5 of Table II) is maximum at 10 to 12 weeks and then gradually decreases to term. The rate at which pyruvate is produced from glucose remains essentially constant from 7 weeks to term (column 6 of Table II). The fact that labelled carbon from pyruvate can be incorporated into glycogen indicates that the glycolytic cycle can operate in reverse,

Table II

METABOLISM OF PYRUVATE BY PLACENTAL SLICES

Age (weeks)	*No. of exp.*	*µmoles/g tissue/hour*			
		Pyruvate produced	*Pyruvate utilized*	*Net pyruvate utilization*	*Pyruvate produced from glucose*
7–9	8	+11·8 ± 1·1*	−19·6 ± 1·7	−7·8	+5·7 ± 0·53
10–12	15	+13·0 ± 0·85	−25·5 ± 1·9	−12·5	+6·9 ± 1·3
13–15	8	+17·2 ± 1·4	−26·6 ± 2·8	−9·4	+6·0 ± 1·1
19–24	7	+19·0 ± 2·9	−27·7 ± 2·7	−8·7	+5·5 ± 0·59
40	22	+14·0 ± 0·55	−22·3 ± 1·1	−8·3	+5·0 ± 0·24

* mean ± standard error of the mean

presumably via glucose-6-phosphate. This is evidence that the inability of the term placenta to secrete glucose is due to the absence of the enzyme glucose-6-phosphatase and not to the failure of some part of the glycolytic cycle.

The production of lactic acid by placental slices was calculated from its rate of appearance in the incubation medium. As with pyruvate utilization, there was considerable variation in the values obtained early in gestation, but there was a gradual decrease in lactate production from 10 weeks to term (Fig. 7).

The source of the lactic acid was determined by experiments in which slices of term placenta were incubated in media containing different substrates (Table III). When no substrate was present in the incubation medium, lactate was produced at a rate of about 5 µmoles/g./hour. This lactate is derived primarily from the breakdown of glycogen, for glycogen disappearance occurred at the rate of 2 to 3 µmoles/g./hour. Each micromole of glucose unit in glycogen yields two micromoles of lactate. The presence of 10 µmoles pyruvate per ml. of incubation medium resulted in the production of an additional 2 to 3 µmoles of lactate per g. per hour. Glucose in the medium at a level of 11·1 µmoles per ml. increased the production of lactate 6 to 7 µmoles per g. per hour over that

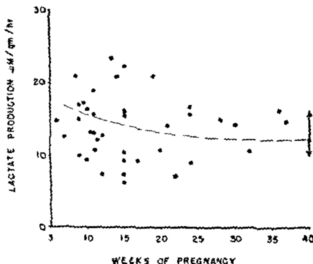


FIG. 7 The production of lactate by the placenta as a function of age. The values are expressed as μmoles per g. of wet tissue per hour. The arrow at 40 weeks indicates the range of values for term placenta.

made when no substrate was added. Since glucose is metabolized to lactate via pyruvate, the greater production of lactate from a glucose substrate than from pyruvate itself can

Table III

THE PRODUCTION OF LACTATE BY SLICES OF TERM PLACENTAS

Gas phase	*Substrate*				
	μmoles of lactate produced per g. tissue per hour				
	None	*Glucose*	*Pyruvate*	*Acetate*	*Oxalacetate*
O_2	4·94	11·9	6·13		
O_2	4·76	10·8	6·84		
O_2	5·11			4·78	3·21
O_2	7·06			6·38	5·03
N_2	11·0	23·3	12·4		
N_2	9·05	21·1	9·58		
O_2	4·50	13·1	8·11		
O_2	4·40	9·7	7·0		

be ascribed to the greater abundance of reduced diphosphopyridine nucleotide produced in glycolysis. Under anaerobic conditions lactate production in the absence of added substrate was about double that found aerobically. Lactate production from glucose was similarly doubled. The increment in lactate production on the addition of pyruvate under anaerobic conditions was small, again suggesting that the limiting factor was the amount of reduced diphosphopyridine nucleotide present. The presence of acetate or oxalacetate, at levels of 10 μmoles per ml., decreased the accumulation of lactate.

Previous experiments with ^{14}C-labelled glycerol had shown that it can be utilized by liver (Tang *et al*, 1953) but not by muscle (Villee, White and Hastings, 1952). When slices of term placenta were incubated with ^{14}C-labelled glycerol, it was found that the placenta is unable to utilize glycerol in glycolysis (Villee, 1953*b*). The evidence favoured the conclusion that term placenta lacks the enzyme which phosphorylates glycerol to form α-glycerophosphate, the first step in its metabolism.

Effects of Hormones

In another series of experiments, tests were made of the ability of slices of term placenta to respond to hormones added *in vitro*. The presence of insulin (0·5 unit per ml.) increased the utilization of glucose and the synthesis of glycogen; it had no effect on the utilization of oxygen or the production of lactic acid (Villee, 1953*b*). Insulin also increased the rate of the utilization of glucose by slices of decidua from a six-week pregnancy and by slices of hydatid mole (pure chorionic villi). The addition of insulin to slices of early placentas increased the rate of glucose utilization to such an extent that the net production observed in the control was changed to a net utilization of glucose in the vessels to which insulin was added. The addition of cortisone or of an aqueous adrenal extract decreased the utilization of glucose and oxygen.

Term placenta also retains its ability to respond to oestrogens added *in vitro* (Villee and Hagerman, 1958). The effect of oestradiol-17β or oestrone has been shown to be a stimulation of isocitric dehydrogenase, an enzyme which requires diphosphopyridine nucleotide (DPN) as coenzyme (Villee, 1955; Villee and Gordon, 1955; Gordon and Villee, 1955).

The Metabolic "Ageing" of the Placenta

From the present experiments it may be concluded that in all of the metabolic functions tested, the activity of the placenta decreases as gestation proceeds. If we may equate decreasing metabolic activity with ageing, then the placenta undergoes ageing. The changes observed include: an increase in the solid fraction of the tissue, a decrease in oxygen consumption to about half the rate earlier, a marked decrease in the glycogen content of the tissue, a loss of the ability of the placenta to synthesize glycogen *in vitro*, a decrease in the rate of glucose utilization, a loss of the ability to produce glucose, and less marked decreases in the rates of pyruvate utilization and lactate production.

A calculation of the fraction of the respiratory CO_2 derived from the labelled substrate, obtained by comparing the specific activities of the centre well CO_2 and the substrate, showed that there was no change in this. The percentage of CO_2 derived from the glucose of the medium was $3 \cdot 7 \pm 0 \cdot 6$ in the early (10 week) placentas and $4 \cdot 0 \pm 0 \cdot 76$ at term. The percentage of CO_2 derived from the pyruvate of the medium was $20 \cdot 8 \pm 1 \cdot 34$ in the early placentas and $18 \cdot 1 \pm 1 \cdot 03$ at term. Thus, although the absolute amounts of glucose and pyruvate metabolized by term placentas were decreased, the relative proportion of the CO_2 derived from each of these substrates was unchanged.

Eight experiments have been performed with placentas from women with toxaemia. The ages of the placentas ranged from 32 weeks to term. In all respects tested, the utilization of glucose, oxygen, glycogen, and pyruvate the

production of lactate and CO_2, and in initial glycogen and water content, these placentas did not differ significantly from normal placentas of a comparable age. These results confirm and extend those of Hellman, Harris and Andrews (1950).

The data presented make it clear that the decreases in metabolic rate are not uniform. Some functions decrease more rapidly, and begin at an earlier time, than others. Glycogen synthesis appears to be one of the first to decrease markedly.

The term placenta, as judged by its metabolic activities *in vitro*, is still quite active. It would seem inaccurate to speak of it as "senile". There is, furthermore, no sharp change at term which could be used to explain the initiation of labour. One could postulate, perhaps, that labour is induced when some critical ratio between the demands of the child for nutrients and the ability of the placenta to supply them is exceeded. The change in the ratio would be brought about, however, more by the rapid increase in the demands of the child as its mass increases than by the decrease in the ability of the placenta to supply nutrients.

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DISCUSSION

Huggett: The thing that I was interested in is this question of glucose production. It would seem that glucose can be produced metabolically within the placenta, and it would be desirable to distinguish that glucose, if it goes into the foetal blood, from the glucose which is passed from the mother to the foetus. So far as one can see that must be going on right away at full term when the metabolic production of glucose within the placenta ceases. Now the point that interests me there is that in the case of the sheep where we have been able to study the passage of sugars across the placenta, this passage of glucose from the mother to the foetus can be reversed by reversing the gradient, but it is not in proportion to the gradient. The amount that moves is more than can be accounted for by the gradient, and equilibrations do not take place. We have to postulate that there is a cellular activity by the sheep placenta in regard to the passage of that particular monosaccharide. So there are two things: the metabolic production and the transmission. I do not know if Dr. Villee would agree with that, but it seems to me a point that we have to keep clear in any analysis.

Villee: I would certainly agree with that, and I think there is no necessary correlation between the two, they may be quite different processes. When I am speaking of the metabolic production of glucose I am, of course, referring to the production of new glucose molecules from something else, and its actual secretion into the incubation medium where the aliquots are taken. Now the transport of glucose, either into a cell or across or through a cell, may be an entirely different process, one which does not involve the formation of a phosphorylated glucose intermediate. Of course, in the human being as well as in the sheep, glucose is obviously passing across the membrane, and I think it is in part, at least, by some active process though not necessarily one which involves phosphorylation.

Huggett: The only other point I would emphasize is the interesting finding that there is no difference between normal and toxaemic placentas. That is rather unexpected.

Villee: You may remember that Hellman was unable to find any

differences between normal and toxaemic placentas either. He had 3 or 4 toxaemic placentas, and as you know they are not too easy to

glucose in any species.

stimulate an extra production of glucose?

Villee: I do not know if we can speak in terms of need. I think Prof. Wislocki mentioned the histochemical evidence that the foetal

Villee: I really do not know.

Williams: What happens, for instance, in a diabetic pregnancy?

Villee: We have tested quite a few diabetic placentas and they were no different from the normal ones.

Williams: But, of course, they are controlled anyway by the insulin

incorporation into glycogen? Does the glycogen decline result from higher rate of breakdown or slower rate of synthesis?

Villee: Early in gestation there is a very active incorporation of

radioactive glucose into glycogen, whereas at term this occurs at a rate which is just barely detectable. I think this is primarily the result of a decreased rate of synthesis rather than an increased rate of breakdown.

Huggett: I was going to ask whether Prof. Jost could advise us whether the foetal endocrines affect placental metabolism. Do you know whether they actually act on the placental metabolism as apart from the foetal organ metabolism?

Jost: I know of no evidence in this field. It has been assumed that the placental glycogen is necessary for the foetus until the time the liver takes on the same function; such an assumption might suggest that there is some correlation between the changes occurring in the placenta and in the liver of the foetus itself. Some results obtained on decapitated rabbit foetuses do not support this idea since the placental glycogen drops even when the liver does not store glycogen. But the foeto-placental endocrine relationship needs further study.

Dempsey: I want to comment on the reference point which Dr. Villee used for the determinations he made. You refer your determinations finally to the dry weight of the slice?

Villee: Only for oxygen, as it is generally done that way. The rest of the determinations are referred to the fresh weight of the tissue.

Dempsey: In either case it is a reference to a weight determination. But isn't there a considerable change in the solid components of the placenta as it goes on to term? My impression is that there is considerably more collagen, for example, in the placenta at term than there would be in some of the earlier placentas.

Villee: The amount of solids goes from about 8 per cent up to about 13 per cent of the total weight.

Dempsey: And some of that solid must be metabolically inert.

Villee: Yes.

Dempsey: So you have as your reference a shifting scale. Would it be possible to have for reference some other determination, which is correlated more sharply with the viable metabolically active tissue, with the living cells, for example, nucleic acid, phosphorus, or something of that sort? Would you think that this would be as good or a worse reference point than the weight?

Villee: It would be at least as good, and perhaps better. Since these changes are gradual and not particularly marked, I have plotted them both as wet weight and as dry weight, and the changes are evident in either case.

Dempsey: Yes, but I was thinking that if there is an accumulation of metabolically inert solid material during the course of pregnancy, then the changes in the determinations which you observed would actually be greater than your figures show, would they not?

Villee: That would be so if our slices were representative of the whole placenta including the collagen. Of course, as collagen is a little difficult to slice we try to find a villus as free of this connective tissue as possible.

Dempsey: Even in the terminal villus, one of the very thin areas, which I think Prof. Wislocki showed on the screen a little while ago,

there is a considerable number of collagen fibres. So I do not think you can avoid it that way.

Villee: No, all we can do is minimize it.

I am sure that there are a great many functions which persist at a

have to do mostly with the release of energy, and I felt that if we did

radioactive glucose into glycogen, whereas at term this occurs at a rate which is just barely detectable. I think this is primarily the result of a decreased rate of synthesis rather than an increased rate of breakdown.

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there is a considerable number of collagen fibres. So I do not think you can avoid it that way.

Villee: No, all we can do is minimize it.

aged, because I think one might also say that an 80-year-old man can still do a great many things.

I am sure that there are a great many functions which persist at a perfectly normal rate in an aged organism. That is, there are some

what we mean by ageing in the case of the placenta.

stages of pregnancy in rats and guinea pigs. A series of goats, pregnant between 35 and 142 days, were subsequently used for further experiments and the relevant results, together with those on the rodents, are given below. The collection of material from a series of goats allowed histological and histochemical observations to be made on the changes occurring in the placenta during the advancement of pregnancy. These observations are briefly summarized below.

Materials and methods

Female albino rats weighing between 170 and 290 g. were used; the animals were killed at times varying from the 11th day of pregnancy until term. Female goats, mainly of the British Saanen breed, were used; the goats were mated at known times and were killed at intervals from the 35th day until just before term. The portions of the uteri and placenta not used for the estimation of radioactivity were fixed in various fluids for subsequent histological and histochemical examination by a variety of methods.

Irradiated "Specpure" K_2CO_3 was converted into an aqueous solution (2 per cent or 0·2 per cent w/v) of ^{42}KCl, as indicated by D'Silva and Neil (1951). In rats the amount of K ion injected in each experiment was 0·3 to 1·2 mg., and in goats 60 to 90 mg., an amount insufficient materially to alter the total amount of K in the extracellular fluid. Descriptions of the technical procedures carried out in order to estimate the radioactivity of the tissues will be found in the paper by D'Silva and Harrison (1953). The potassium content was determined by means of a direct reading flame photometer.

Experimental results

In a series of rats pregnant for 11 to 18 days the distribution of ^{42}K in the uterus, placenta, kidney and foetuses was studied 5 minutes after intravenous injection of ^{42}KCl. It will

be seen from Table I that with the smallest foetus (<9 mm.) the radioactivity in the uterus was less than that in the placenta. When the foetus was 9–18 mm. long the reverse was true. The results of a larger number of experiments, carried out near term and involving the sacrifice of the animals

Table I

THE ACTIVITY OF TISSUES IN THE PREGNANT RAT 5 MINUTES AFTER INTRAVENOUS INJECTION OF ^{42}KCl

Rat No.	*Duration of pregnancy in days*	*Foetal length (mm.)*	*Kidney*	*Uterus*	*Placenta*	*Foetus*
55	11	6	5·74	0·825	1·13	—
41	13	6	3·41	0·463	0·512	—
35	14	7	6·68	0·400	0·910	0·022*
34	14	8	3·15	0·313	0·391	0·030*
43	14	9	3·33	0·509	0·303	—
44	14	9	2·98	0·501	0·315	—
63	18	9	4·94	0·660	0·545	—
36	14	10	4·32	1·140	0·689	0·015*
37	14	10	5·34	0·737	0·601	—
46	15	10	4·03	0·565	0·322	—
38	15	12	5·48	0·822	0·635	—
39	15	12	5·27	0·693	0·509	—
61	17	17	5·51	0·568	0·350	—
60	17	18	4·01	0·590	0·383	—
Mean S.D.	— —	— —	4·59 ±1·14	0·630 ±0·210	0·545 ±0·242	—

* Includes foetal fluids.

Activity of uterus, placenta and foetuses at various times after injection of ^{42}KCl into rats. Results are expressed as percentage (x) of total radioactivity x is defined by the expression,

$$x = \frac{\text{counts/min /g. tissue}}{\text{total injected counts/min}} \times 100\ D$$

where D is a correction factor allowing for decay.

at intervals of 5 minutes to 24 hours after injection of ^{42}KCl are described in the paper by D'Silva and Harrison (1953). The activity of the labyrinthine placenta near term falls from 0·53 per cent (per g. of tissue) of the total radioactivity after 5 minutes to 0·20 per cent after 24 hours. It is suggested that these results could be explained on the assumption of a slow

rate of blood-flow through the uterine vessels (Barcroft and Rothschild, 1932; Barcroft, Herkel and Hill, 1933) together with a rapid rate of exchange of K ions.

A further series of experiments was carried out on goats of various breeds, pregnant from 35 to 142 days, into which radioactive ^{42}KCl was injected 15 minutes before death. In almost every animal portions of the kidney and liver were removed and the radioactivity determined. In all but two animals the activity of the plasma was also determined and this allowed calculation of the relative potassium activity (R.P.A.). The latter is calculated according to the following expression:

$$\text{R.P.A.} = \frac{\text{Activity of tissue}}{\text{Activity of plasma}} \times \frac{\text{mg.K/g. of plasma}}{\text{mg.K/g. of tissue}}$$

where the activity is defined by the expression given in the footnote to Table I.

Table II shows that the radioactivity of both the kidney and liver varied widely in different animals, but there was no systematic change in relation to the duration of pregnancy. On the whole, the radioactivity of the tissue was directly related to its potassium content which is shown by the relatively small variation in the figures for the R.P.A. of kidney (column 7) and of liver (column 10).

The membranous chorion was removed from portions of the uterine wall and areas of mucosa carefully stripped from the underlying muscle. The radioactivity and K content of the uterine muscle and mucosa are shown in Table III. There were large changes in the radioactivity of these tissues in different experiments but these were roughly paralleled by changes in K content. Therefore the R.P.A. values for uterine muscle (column 5) and mucosa (column 8) were much less variable than either the radioactivity or the K content.

In each animal either a whole placentome, or a portion of one, was removed and examined for its activity and its potassium content. In several animals a number of placentomes of varying shape and size were examined individually;

there was no significant difference in their activity. Different slices (about 0·5 g.) of the placentome gave results similar to those obtained from a whole placentome removed from the same animal. The activity of the placentome was 0·0058 per

Table II

THE ACTIVITY OF TISSUES IN THE PREGNANT GOAT 15 MINUTES AFTER INTRAVENOUS INJECTION OF ^{42}KCl

Goat no.	*Days preg-nant*	*Plasma*		*Kidney*		*R.P.A.*	*Liver*		*R.P.A.*
		Activ-ity[1]	*mg.K*[2]	*Activ-ity*[1]	*mg.K*[2]		*Activ-ity*[1]	*mg.K*[2]	
52	35	0 0012	0·17	0·018	2·31	1 10	0·008	3·30	0·35
6	39	0·0016	0·14	0·023	2·72	0·75	0 013	3 60	0·30
7	45	0·0012	0·40	0 014	3·10	1·45	0·009	4·13	0·72
59	45	0·0014	0·14	0·011	1·32	0·83	0·013	3·17	0·59
37	59	0·0018	0·16	0 025	2·20	0 98	0·010	3·20	0·27
9	66	0 0025	0 36	0 018	2·68	1·02	0·012	4·45	0·41
14	75	—	—	0·022	—	—	0·012	5·50	—
12	87	0·001	0·39	0 010	2·72	1·40	0·006	4·97	0·47
46	91	0·0013	0·15	0·020	2 90	1·15	0·016	3·87	0·48
58	92	0·0011	0·24	0·015	2·50	1·30	0·014	3·26	0·94
13	96	—	—	0·010	2·82	—	0·004	3·50	—
32	100	0·0011	0·22	0·017	2·71	1·20	0·010	3·41	0·30
36	107	0 0019	0·18	0·019	2·40	0·75	0·012	3·56	0·32
38	116	0·0022	0·14	0·044	3·15	0·80	0·030	3·72	0·52
1	123	0 0047	0·22	0·011	2·00	0·26	—	—	—
48	125	0 0021	0·18	0·020	1·96	0·84	0·016	4·10	0·34
18	136	0·0015	0·28	0 023	3·05	1·40	0 015	3·47	0·83
20	142	0 008	0·19	0·014	3·82	0·84	0·008	5·07	0·37
21	142	0·0012	0·16	0·024	3·11	1·00	0·016	5·81	0·37
Mean S.D.						1·01 ±0·30			0·48 ±0·20

[1] Activity determined as shown in footnote to Table I.
[2] Potassium content expressed as mg of K per g. of wet tissue

cent at the 35th day and 0·0083 per cent of the total injected radioactivity at the 142nd day of pregnancy. The average activity of the placentome throughout pregnancy was 0·0099 per cent; the lowest figure being 0·0023 per cent on the 92nd day and the highest 0·032 per cent on the 91st day. The

extreme figures were associated with a correspondingly decreased or increased potassium content. There is thus no evidence that the uptake of radio-potassium per g. of placentome 15 minutes after injection varies during pregnancy.

Table III

The Activity of Tissues in the Pregnant Goat 15 Minutes after Intravenous Injection of ^{42}KCl

Goat No.	*Days Pregnant*	*Uterus*		*R.P.A.*	*Mucosa*		*R.P.A.*
		Activity[1]	*mg K*[2]		*Activity*[1]	*mg.K*[2]	
52	35	0·0063	2·26	0·39	0·010	2·06	1·30
6	39	0·0076	2·00	0·23	—	—	—
7	43	0·006	2·71	0·63	0·008	2·69	0·98
59	45	0·0043	2·26	0·19	0·007	2·06	0·34
37	59	0·0065	2·04	0·28	0·015	2·12	0·63
9	66	0·004	2·87	0·21	0·007	2·11	0·51
14	75	0·004	2·87	—	0·012	2·06	—
12	87	0·004	1·86	0·84	0·0037	2·20	1·01
40	91	0·004	1·05	0·44	0·015	1·61	1·04
58	92	0·0033	2·71	0·26	0·0082	2·40	0·74
13	96	0·0024	3·33	—	0·0015	3·10	—
32	100	0·0036	2·88	0·39	0·0082	4·49	0·37
36	107	0·0027	2·77	0·09	0·0036	2·13	0·34
38	116	0·017	2·16	0·50	0·029	3·02	0·61
1	125	0·009	1·70	0·24	0·020	1·90	0·50
48	125	0·0075	2·93	0·21	0·021	3·80	0·47
18	136	0·008	2·52	0·59	0·002	2·03	0·18
20	*142*	*0·0037*	*6·20*	*0·14*	*0·0058*	—	—
21	142	0·006	3·39	0·24	0·010	2·84	0·47
Mean				0·35			0·63
S.D.				±0·19			±0·32

[1] Activity determined as shown in footnote to Table I.
[2] Potassium content expressed as mg of K per g of wet tissue.

The *membranous chorion* was rich in potassium, but the quantity present was usually less than that in the placentome (per g. of wet tissue). The radioactivity of the membranous chorion was less than that of the placentome, except in Goat 14, where it was slightly higher. The activity of the membranous chorion per g. was 0·0004 per cent at the 35th day,

and 0·001 per cent of the total radioactivity at the 142nd day of pregnancy. The average of all the estimations of the activity of the chorion was one fifth of that of the placentome 15 minutes after injection. The R.P.A. of the membranous

Table IV

THE ACTIVITY OF TISSUES IN THE PREGNANT GOAT 15 MINUTES AFTER INTRAVENOUS INJECTION OF ^{42}KCl

Goat No.	*Days preg-nant*	*Placentome*		*R.P.A.*	*Membranous chorion*		*R.P.A.*
		Activ-ity[1]	*mg.K*[2]		*Activ-ity*[1]	*mg.K*[2]	
52	35	0·0058	1·35	0·61	0·0004	0·96	0·06
6	39	0·0074	1·47	0·44	0·0013	1·47	0·08
7	45	0·013	4·19	1·02	0·00045	1·42	0·11
59	45	0·0077	2·20	0·35	0·00097	2·39	0·04
37	59	0·010	2·53	0·35	0·0038	1·11	0·32
9	66	0·006	3·58	0·33	0·00063	1·02	0·10
14	75	0·006	2·36	—	0·011	2·37	—
12	87	0·0044	2·78	0·63	0·00034	2·54	0·03
46	91	0·032	7·30	0·44	0·0013	1·45	0·10
58	92	0·0023	1·84	0·27	0·0014	1·58	0·19
13	96	0·0025	3·15	—	0·00036	1·03	—
32	100	0·0074	5·76	0·26	0·0029	1·90	0·31
36	107	0·009	—	—	0·0004	1·55	0·05
38	116	0·017	2·80	0·38	0·0020	1·30	0·13
1	125	0·023	2·50	0·48	0·0028	1·36	0·09
48	125	0·013	2·68	0·41	0·0014	1·71	0·07
18	136	0·009	2·23	0·76	0·0011	1·49	0·14
20	142	0·005	2·30	0·48	0·00049	5·40	0·02
21	142	0·0083	3·07	0·36	0·0011	2·87	0·03
Mean S.D.				0·45 ±0·22			0·11 ±0·09

[1] Activity determined as shown in footnote to Table I
[2] Potassium content expressed as mg. of K per g. of wet tissue

chorion was considerably less than that of the placentome, which suggests that the chorion exchanges its K at a slower rate than the placentome. It is possible that this difference is due to the lack of opportunity for exchange between the blood and the cells of the chorion.

Morphological observations

The experimental observations outlined above made available material from a series of animals killed after a known period of pregnancy. Material from other goats, also of known gestation age, has resulted in the examination of over sixty animals killed at different stages of pregnancy.

Recent papers on the developmental changes in the placenta of the Artiodactyla include those by Wimsatt (1950, 1951), Harrison and Hamilton (1952), Björkman (1954) and Harrison and Hyett (1954). A full review of earlier papers on the placenta of the cow, sheep and pig has been given by Amoroso (1952).

The placenta of the goat is polycotyledonary with from 120 to 180 cup-shaped or concave placentomes (Pl. I, Fig. 5) arranged more or less in rows. The placentomes on the mesometrial aspect of the central portion of each uterine horn are, for the first third or more of pregnancy, substantially the larger. As pregnancy advances the cranially placed placentomes increase more rapidly in size than those centrally placed, but do not reach a comparable size. Occasionally flattened, sessile, oval placentomes are encountered together with the concave variety, and in several animals killed during the middle of pregnancy only the flattened, oval variety was present. It should be noted that the uptake of radioactive potassium by the two types of placentome was not significantly different. It is not yet clear if this morphological difference is genetic in origin, or a manifestation of change in the form of the placentome during development.

Primary villi are present and have penetrated deep into the caruncular tissue even by the 39th day of pregnancy (Pl. I, Fig. 4). The primary villi are at first straight and simple, but active division at their tips results in the formation of secondary and tertiary villi, each fitting into a corresponding maternal crypt. From the earliest stages studied (35th day) it appears that the trophoblast has considerable powers of attrition and the maternal epithelium is destroyed not only in the crypt walls but also in localized areas in relation to the

membranous or intercotyledonary chorion (Pl. I, Figs. 1 and 3). Near the edges of the developing caruncles the chorion is thrown into folds or intermediate villi, the areas thus resemble the arcade formations described by Assheton (1906) and Björkman (1954). The maternal epithelium also shows signs of impending degeneration in the septal walls surrounding the intermediate villi (Pl. I, Fig. 2). Only in the regions of the mouths of the uterine glands does the maternal epithelium display the characteristics of healthy tissue, and where the chorion comes into contact at the edges of the gland mouth localized areas of cellular attrition are present (Pl. I, Fig. 3).

The powers of attrition apparently possessed by the chorionic epithelium may be reflected by the increase in density of the sub-epithelial stratum compactum to a degree greater than that observed by Hamilton and Harrison (1951) in the non-pregnant uterus of the goat. This increase in density appears to be due not only to changes in the cellular population but also to increase in the quantity of connective tissue and changes in its staining qualities and also in those of the intercellular substance. It is noticeable that the destruction of maternal tissue attributable to the chorion is capable of causing localized haemorrhages between foetal and maternal tissues. Such haemorrhages can be seen in early

PLATE I

Fig. 1. Photomicrograph to show destruction of the maternal epithelium during the early stages of pregnancy. The animal had been pregnant for 39 days. Section stained by the P.A.S. method. × 70.

Fig. 2. Photomicrograph to show the formation of folds in the chorion at the edge of the developing caruncle. The animal had been pregnant for 39 days. Haematoxylin and eosin. × 70.

Fig. 3. Photomicrograph of a membranous chorion in relation to the mouth of a maternal gland. The animal had been pregnant for 39 days. Haematoxylin and eosin. × 75.

Fig. 4. Photomicrograph to show the appearance of the young villi in the developing caruncle at the 39th day of pregnancy. Haematoxylin and eosin. × 42.

Fig. 5. The appearances of the placentomes on the 50th day of pregnancy, showing haemorrhages on the surfaces of the placentomes. × ½.

Fig. 6. Photomicrograph of a small accessory placentome on the 60th day of pregnancy, showing an extravasation of blood between the maternal and foetal tissues.

PLATE I.













is frequently to be observed a narrow layer of pyknotic, heavily staining nuclei separating the chorionic cells from the maternal stroma, not only in the region of the membranous chorion (Pl. II, Fig. 1) but also in the placentome (Pl. II, Fig. 5). This layer may represent the attenuated and dying maternal epithelium, but it is also possible that it is the remnants of foetal cells which have invaded and destroyed the maternal epithelium and have themselves subsequently died (Harrison and Hamilton, 1952).

Trophoblastic giant cells, possessing the histochemical characteristics so clearly described by Wimsatt (1951) in the sheep, are observable at the edges of the villi (Pl. II, Fig. 3). Similar cells can also be observed in situations which strongly suggest that the giant cells pass across and become intercalated within or even replace the maternal epithelium. It is, however, difficult to decide whether these cells can exist for very long in their close relationship to the maternal stroma.

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DISCUSSION

Wislocki: I should like to ask Prof. Harrison whether, besides the *chorioallantoic placenta, he also investigated potassium transfer through* the yolk-sac placenta, and what differences he found?

Harrison: We made a few estimations of that. The uptake after 15 minutes and one hour were negligible. The uptake was so small that it was not significant in comparison with the uptake of other parts of the placenta. And that frankly did surprise us. I would have expected that there would be considerable uptake, but there was not—but that was not near term and it may be that that is relevant to Prof. Dempsey's comments earlier on.

Huggett: There are two points. First, Cloette observed in the

The second point is, Dr. Seoras Morrison has been working with me during the last few months on the uptake of [^{14}C]glucose, given to the mother, by glycogen in the rabbit placenta and there we find quite definite differences between the decidual glycogen and the chorionic glycogen. They do not behave in the same way. I was also very interested in the general finding you had that the placenta compared with the liver has a very latent active uptake—I hope I may use that term.

Harrison: Yes—the cup-shaped placentomes have a potassium content and an uptake of radioactive potassium equivalent to that in the flattened placentomes. Both liver and placentome are rich in potassium, but the uptake of radioactive potassium by the placentome is not as great, therefore one thinks of differences in the blood supply, and the rate of flow and thus the opportunity for exchange.

Strauss: I wonder if Prof. Harrison found any potassium in the walls of the foetal blood vessel of the placenta. A couple of years ago we found some potassium in human mature placentas. The potassium is in the syncytiotrophoblast as well as in the walls of the foetal blood vessels. We found about the same distribution of the calcium. Have you any experience of that?

Harrison: We have not done any quantitative estimations on that. We have only made some autoradiographs, and one can detect the potassium in the chorion, but we have not looked carefully or made any comparison with the amount in the walls of the blood vessels. We have estimated the uptake in the umbilical cord and that is insignificant compared with the uptake by the placentome. So it was from that experiment that we thought we could argue that the connective tissue in the placenta did not take up potassium to a degree parallel to the chorionic epithelium.

Huggett: You are not distinguishing between Wharton's jelly and the vessels of the cord?

Harrison: We simply took a chunk of the cord.

Hamilton: How early do the hæmorrhages occur, Prof. Harrison? I have not seen those before in any of the ungulates.

Harrison: That picture I showed was, I am almost sure, 41 or 42 days old, but we found such localized hæmorrhages right from the start of the formation of the placentomes. It is quite different from the state of affairs that we found in the deer. We did not find any hæmorrhages, as far as I can remember, at any stage in any species of deer we looked at.

Hamilton: I take it that the trophoblast is eroding maternal blood vessels in some way or another.

Amoroso: I have no doubt in my own mind that the trophoblast is endowed with erosive properties. That it may destroy the maternal capillary endothelium is a real possibility. As to the question of the two types of placentomes to which Prof. Harrison has drawn our attention, these have been figured in the uterus of the sheep by Cloette and

degradation.

Montagna: A great deal has been said about the efficiency of the

judge of efficiency.

Amoroso: Are you suggesting that it is the quality of the embryo at the end of gestation which is the criterion?

Montagna: I am saying that the placenta is always efficient if the embryo is going to survive.

MODIFICATIONS IN THE FOETAL DEVELOPMENT OF THE RAT AFTER ADMINISTRATION OF GROWTH HORMONE OR CORTISONE TO THE MOTHER

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"The art of living," said the Greeks, "consists in dying young, but as late as possible." This, surely, is the goal that gerontology has set for itself.

When one considers the problem of ageing, which is among the most fascinating for the biologist, one question at once arises: are the development, functioning and evolution of *the organs in the course of life unalterably fixed by heredity* or do they also depend to a certain extent on internal and external factors over which we may have some influence? The second alternative is the more probable, if not the more desirable. The increase in longevity which we have witnessed constitutes an encouraging argument in this respect.

For the past century it has been affirmed that the endocrine glands are capable of delaying or hastening ageing, and it is *this idea that has been the basis of hormone therapy* and the great advances in endocrinology. If the over-optimistic theories of the early workers in endocrinology have unfortunately not proved correct, it is nevertheless probable that the endocrine balance does play an important part in the growth, functioning and ageing of the organs.

Numerous authors have carefully analysed the morphological processes in the ageing of the endocrine glands. Loeb (1941), who has made a very detailed study, has described the progressive modification of the different endocrine glands with age; he has also shown that depending on the

experimental conditions certain hormones, such as the oestrogens, are capable of delaying or, on the contrary, of hastening the ageing of the endocrines. It seems, therefore, as if modifications in hormonal equilibrium are capable of influencing the general process of development and ageing. During the last two years we have tried to examine the problem of the relationship between the endocrine glands and the somatic development by modifying the endocrine balance of the pregnant female. The results of two series of experiments are given here, one on the action of the somatotrophic hormone (STH), the other on that of cortisone.

Somatotrophic Hormone

The well-known part played by the anterior pituitary in somatic development has led many authors to consider somatotrophic hormone as a stimulant not only of postnatal growth but also of embryonic development. That, at least, is the conclusion reached by Teel (1926), Hain (1932), Sontag and Munson (1934) and Watts (1935), who observed that the injection of crude extracts of the anterior pituitary into pregnant rats gave rise to foetal gigantism. Using the purified hormone Hultquist and Engfeldt (1949), Engfeldt and Hultquist (1953), Nixon (1954) and Cotes (1954) also produced in rats enormous foetuses weighing up to 7 and 8 g. However, the injection of STH into pregnant females of other species, such as the dog and the cat, gave inconsistent results (Young, 1946).

The question of the foetal gigantism obtained with STH has often been raised in connection with the large children born of diabetic mothers, and highly ingenious interpretations have been put forward to explain the exaggerated foetal growth. However, it seems improbable that STH, due to its high molecular weight, could cross the placental barrier, and the possibility of stimulating embryonic growth with STH seemed astonishing, and therefore we repeated the experiments on the rat.

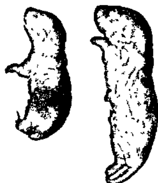


FIG. 1. Newborn rats; left, control; right, offspring of an STH-treated mother.

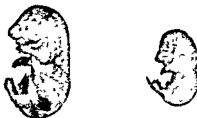


FIG. 2. Rat embryos removed on the 20th day of gestation. Left, control; right, embryo of STH-treated mother.

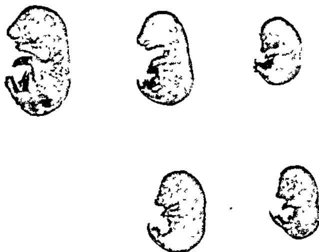


FIG. 3. Above: left to right, embryos of control rats at the 20th, 19th and 18th days of gestation; below: two 20-day embryos removed from STH-treated mothers.

revise our ideas of "foetal gigantism of pituitary origin". In actual fact, if one compares the weight of the newborn of STH-treated mothers which are born between the 24th and 28th day of pregnancy with that of young rats of the same actual age, that is to say two to six days post-partum, one observes that the latter are always the larger. The greater

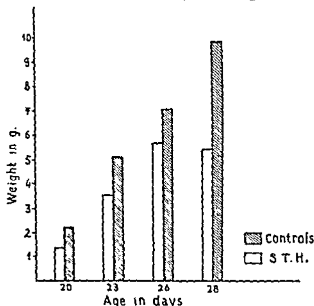


FIG. 5. Comparison of the weights of the offspring of STH-treated mothers with those of control rats of the same age.

weight of the controls is, as shown in Fig. 5, of the order of 40–50 per cent.

Thus when one compares the weight of the foetuses, taking into account not the weight at birth but at their actual age, one observes that the increased weight of the newborn is in fact merely an apparent one and due solely to the prolongation of intra-uterine life. Contrary to the generally accepted opinion, the administration of somatrophic hormone to the mother not only does not stimulate the embryonic

growth of the rat but inhibits it and makes it possible to obtain embryos of younger appearance than the normal embryos of that age.

These observations led us to examine two other aspects of this problem: the possible mechanism of the action of STH and the part played by the maternal pituitary in embryonic development. The causes of the inhibition of embryonic development observed with STH are difficult to explain. We

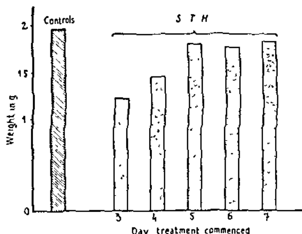


FIG. 6. Average weight of embryos removed on the 20th day of gestation; the differences in weight are only significant when STH is administered before the 5th day of gestation

were tempted to explain it by an increase of maternal protein anabolism. This could in fact deprive the foetus of part of the nutritive substances intended for it. However, a complementary experiment, in which we commenced STH administration 4, 5, 6, 7 and 8 days after conception, showed us that foetal growth is inhibited to a significant degree only when STH is administered before the 5th day of gestation (Fig. 6). This fact suggests another mechanism, for example an early enzymatic disturbance, which has a deleterious effect on the development of the embryo, as has been clearly shown by the work of Warkany (1948) and Giroud (1954).

With regard to the problem of the influence of the maternal pituitary on the development of the embryo, our experiments with hypophysectomy on the 12th day of pregnancy, that is, when the placental secretion is sufficient to make up for the hypophyseal gonadotrophins, show that hypophysectomy at this stage is generally well tolerated, and we had only a few postoperative abortions. The progress of pregnancy is in most cases normal. The foetuses removed on the 20th day are normal and their weight identical with that of embryos from control rats which had undergone a spurious operation. The average weight of the offspring of hypophysectomized mothers is 2·12 g. as compared with 2·20 g. for the controls. Our results confirm those of Campbell, Innes and Kosterlitz (1953), and in no case have we observed any diminution in weight like that reported by Knobil and Caton (1953). Moreover, when pregnancy is allowed to proceed to term and after, it is observed that foetal growth continues. With the exception of two rats which gave birth on 22nd–23rd day to live foetuses of normal weight, the others showed difficulty in giving birth and we had to have recourse to artificial deliveries between the 24th and 25th day in order to recover the foetuses. It is interesting to note, on this point, that with the prolongation of gestation, the weight of the foetuses increases even in the absence of the pituitary, and that those removed on the 24th day weigh 5·6 g.

Discussion

These observations show that the development of the embryo is relatively independent of the pituitary control. When one compares the results of our experiments with hormone therapy and hypophysectomy, one is led to conclusions which are directly opposed to frequently expressed *opinions on the part played by somatotrophic hormone in* embryonic growth. In point of fact, taking as a basis the experiments with STH administration in course of which foetal gigantism was thought to be observed in the rat, Young (1953) admits that the somatotrophic activity of the pituitary

is increased during gestation. Moreover, the author believes that the foetal gigantism which occurs in certain pathological conditions, notably with diabetic and pre-diabetic mothers, could be brought about by a somatotrophic hypersecretion of the maternal pituitary.

However, the somatotrophic hypersecretion in the pregnant female, postulated by Young and often accepted by clinicians, has not been demonstrable. The results of our experiments make the suggestion that the influence of STH is the cause of foetal gigantism, hardly credible. Moreover, the fact that maternal hypophysectomy does not inhibit foetal development also shows that embryonic growth is not pituitary-controlled. This interpretation is supported by the fact that the destruction or ablation of the foetal pituitary (Raynaud and Friley, 1947; Jost, 1947) does not appear to slow down foetal growth in the mouse or rabbit. It also links up with *clinical observations on the children of acromegalic mothers.* Neither Jackson (1954) nor Huant (1955) have observed the stigma of gigantism in the children of acromegalic mothers; in one case, Huant even noted signs of marasmus and under-development.

Thus these experimental results and clinical observations confirm each other and that somatotrophic hormone does not enter into the embryonic development of mammals.

Nevertheless, the inhibition of the embryonic growth of the rat, which we observed with early administration of STH and which is probably indirectly caused, is worthy of our attention, for the possibility of consistently obtaining embryos of more juvenile appearance may provide an interesting method of studying the metabolic factors conditioning embryonic growth.

Cortisone

The inhibition of embryonic growth by somatotrophic hormone led us to examine the influence of another hormone, cortisone. It is remarkable that whilst somatotrophic hormone

increases protein anabolism, cortisone increases protein catabolism.

When pregnant rats are treated from the 6th to the 16th day of pregnancy with cortisone in doses of 20 mg. per day, disturbances of gestation and delivery are observed. Courrier and co-workers (1951), like Robson and Sharaf (1952), have similarly observed in the rat and the rabbit disturbances of gestation, abortions and difficulties in delivery under the influence of cortisone and ACTH.

With the doses of cortisone which we have used (Tuchmann-Duplessis and Mercier-Parot, 1954), abortions in our breeding rats are relatively rare. The foetuses are, in the majority of cases, live, of normal appearance and their average weight is only 10 per cent lower than that of the controls. We have not observed *malformations like those reported by Fraser, Fain*stat and Kalter (1953) in mice.

Nevertheless, in spite of their normal appearance, these newborn rats are extremely frail. Although appearing to nurse normally, that is their milk intake is normal, the majority of the young rats die between the 3rd and 4th day. The growth of the rare survivors is, as shown in Fig. 7, considerably slowed down or completely arrested. Sickly, rapidly becoming senile and wrinkled in appearance, these animals do not generally survive for more than 12 to 18 days.

Thus, if the administration of cortisone to the pregnant rat only slightly delays embryonic development, it almost completely arrests postnatal growth.

Two series of experiments were carried out in order to investigate the possible causes of the inhibition of postnatal growth: (1) the exchange of newborn between cortisone-treated mothers and control mothers, and (2) the treatment of control mothers with cortisone after parturition.

In the first series of experiments out of 10 attempts we have only succeeded so far with one exchange of newborn rats. However, the result seemed noteworthy, for, after an initial delay, the few newborn from the cortisone-treated mother and reared by the control mother, developed relatively well.

Conversely, the offspring of the control mother suckled by the rat treated with cortisone during gestation, are retarded in growth and show dorsal alopecia as well as markedly delayed sexual development. Although in both cases the milk intake of the newborn appears to be normal, the result of the

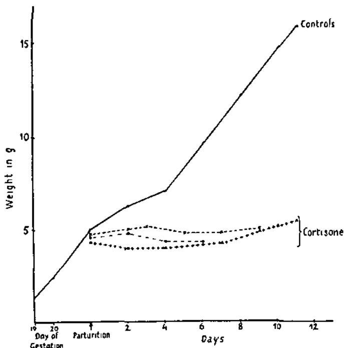


FIG. 7. Growth curves of young rats born of mothers treated with cortisone during pregnancy.

exchange of mothers indicates that cortisone has an unfavourable effect on lactation.

The results on two sets of animals obtained at a six months interval prove this. The rats in the first set received 20 mg. of cortisone for 5 days following parturition and 10 mg. after this; those in the second set received 20 mg. of cortisone for 15 days following parturition. The cortisone treatment of the

nursing mothers considerably delays the somatic development of the newborn and increases their mortality (Mercier-Parot, 1955). The course of the growth of the young rats

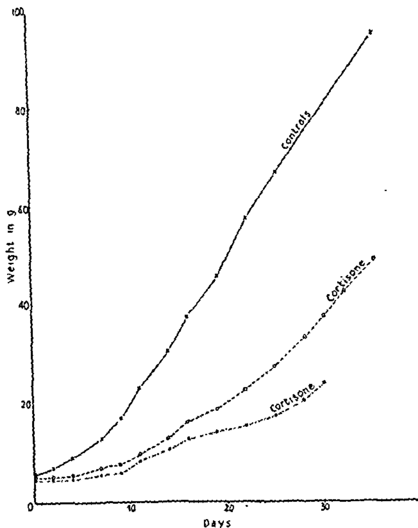


FIG. 8. Growth curves of young rats nursed by mothers treated with cortisone after parturition.

shows two characteristics, as shown in Fig. 8. During the first phase of 4–6 days, the retardation in growth compared with the controls is slight, being of the nature of 15 to 20 per cent. From the 6th day the arrest in development is accentuated and on the 10th day the difference in weight compared with the controls is from 50 to 60 per cent. The majority of the young die between the 12th and the 15th day. Of 50 new-born, 5 reached the age of 30 days and only two have survived two months. Although these two rats partially made up their delay in somatic development during the second month, the genital organs retained their infantile appearance.

Thus at first sight cortisone proves just as deleterious to the growth of young rats when it is administered during lactation as when it is administered during gestation.

Conclusions

The two series of experiments that we have described here show that, in spite of its autonomy, foetal development can be modified by disturbances in the endocrine balance of the mother.

Contrary to the long accepted opinion, it does not seem feasible that foetal gigantism can be accounted for by the maternal somatotrophic secretion. Although hypophysectomy in the mother on the 12th day of pregnancy does not influence the development of the embryos, the early administration of STH unquestionably inhibits the embryonic development. At the same age, the weight of the foetuses of STH-treated mothers is about half that of the controls, and these embryos are more juvenile in appearance. At 20 days the surfaces of the nervous system and of the visceral organs of these embryos have scarcely reached the stage of development of control embryos of 19 days. If the causes of this growth inhibition still remain unexplained, the fact itself is interesting for it opens up new possibilities of research into the metabolic factors capable of influencing somatic growth.

The results of cortisone administration show that, in spite

of the slight difference in weight at birth—10 per cent lower than the controls, the postnatal development of these young rats is greatly inhibited or arrested. Although we have been able to show that this inhibition appears to be connected with disturbances of lactation, the great frailty of the offspring of cortisone-treated mothers seems to indicate the existence of a more or less reversible change in their constitution.

Acknowledgement.

We wish to express our thanks to M. Mémin, for taking the photographs.

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DISCUSSION

Jost: The two kinds of experiments which were shown seem to be rather different. In the first one, pituitary extracts were used and the question immediately arises whether the extracts were completely free of substances stimulating other endocrine glands, such as the adrenals or the ovary. It is well known that maternal hormonal imbalances may

average size of rat foe
pounds to the mother.
on day 21 is about 5
propionate, testostero:

philic cells will directly determine the sexual development of the embryo.

Jost: I wish to introduce a remark about some papers claiming that growth hormone injected into pregnant rats produces foetal gigantism. I must say that a careful examination of the published tables often leads to other conclusions than those expressed by the author in the text accompanying the tables. I know of no clear demonstration of foetal gigantism induced by growth hormone.

T.-Duplessis: No, they got a very large foetus of about 8 g. and they did tremendous work in measuring the different endocrine glands, and where these glands were larger and so forth, they tried to explain that also by growth hormone secretion or pathological aspects, for instance in a diabetic mother. But I think that they were always dealing with older embryos.

Huggett: At the end of the war, Dr. Deryk Fraser and I were interested in foetal growth and the relationship between growth hormone and diabetogenic hormone and the effect on growth gonadally, so we injected growth hormone, which we got from Prof. Frank Young, and we found, as Prof. Tuchmann-Duplessis has found, that the foetuses were smaller. But we also found, as he did, that if you injected not at the beginning of conception but after 5 days, it had no effect, and that gave us the clue and we worked out that in fact what we were dealing with was delayed implantation due to the presence of impurity. We proved this because later Prof. Young produced a very pure growth hormone, completely free of gonadotrophin, and we found that it was completely inactive and had no effect whatsoever.

Like Prof. Jost, we came to the same conclusions on reading some of the published papers—that our conclusions differed from the authors'.

Strauss: I would like to know where the growth hormone came from? Is it a pure hormone or a complex one?

T.-Duplessis: We tried two types of growth hormone, a crystalline one prepared by Li in California and a commercial brand (Choay Laboratories) in which there may have been gonadotrophins. I think we have to bear in mind that these hormones only modify the environment of the mother—they do not cross the placental barrier. It was demonstrated by many people that pituitary hormone, which, like ACTH, has a lower molecular weight does not cross the placental barrier.

Strauss: But if growth hormone *does* contain gonadotrophic hormone, then gonadotrophic hormones may be produced by the placenta itself.

T.-Duplessis: But the results were the same whether you have a preparation which you are quite sure does not contain gonadotrophin or one which does. It is only the mortality or abortion rate which is higher if you have a mixture of both. This is very significant in the experiments reported by Nixon.

Huggett: Swyer and Fouracre Barns also investigated this same problem using pure growth hormone, and got the same result as we got, namely the entire negative effects.

Williams: If growth hormone only acts if it is given before the 5th day, you can conceive that this is the period when pituitary luteotrophin is active and that by inhibiting production of this you delay implantation. If you are giving very high doses of growth hormone you may depress

the pituitary even if the preparation contains no gonadotrophin and it

hair follicle itself begins to grow no growth occurs in spite of the application of stimuli which would normally initiate growth. This is the reason I asked if we are sure that it is due to delayed implantation and not to some other condition.

Huggett: That is quite a valid point.

Jost: I should like to recall how complicated the problem of delayed

Tuchmann-Duplessis' experiments cortisone did not act chiefly after birth.

T.-Duplessis: I do not know why the embryo is protected, though I do not think it is as well protected as it would appear to be, because the

THE GROWTH CYCLE OF DEER ANTLERS

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The antlers of deer are deciduous, being annually renewed. In the Virginia deer (*Odocoileus virginianus*) in its natural habitat, they begin to grow visibly in April or May, reach maturity in August and the velvet is shed in September. The mature antlers, consisting of bare, dead bone, remain in place, firmly attached until mid-winter when they are shed.

Antlers grow by the addition of new material at the extremity of the beam and at the tips of the tines as they arise. The growing tip moves away from the antler pedicle, depositing a column of bone which, once laid down, does not increase appreciably in diameter as it matures. All stages of growth and maturation can be seen in a single growing antler; the bone nearest the antler base is the oldest and most mature, whereas that at the tips is most recent and in process of formation.

Microscopically, the mode of bone differentiation at the tips of the growing antlers is observed to be intermediate, in a number of important respects, between intramembranous and endochondral ossification (Wislocki, Weatherford and Singer, 1947). In the growing tips beneath the velvet, germinal, preosseous and osseous zones are visible. The germinal zone located immediately beneath the velvet consists of large fusiform and stellate cells with extremely basophilic cytoplasm and showing numerous mitoses. The cells of the preosseous zone lie in lacunae contained within a matrix. The matrix consists of a dense meshwork of reticular, collagenous fibres and of a ground substance; the latter is metachromatic following staining with toluidine blue and gives an alkaline phosphatase reaction. Each lacuna is enclosed by a capsule which is

strongly basophilic when stained with methylene blue and intensely metachromatic with toluidine blue, and gives an intense reaction for alkaline phosphatase. The basophilia and metachromasia of the lacunar capsules bespeak the presence of a sulphated acid mucopolysaccharide and a resemblance to cartilage. However, on other grounds, including the presence of a dense meshwork of collagen, the arrangement of the cells and the pattern of the blood supply, the preosseous tissue differs markedly from ordinary hyaline cartilage and endochondral bone formation as seen in the growing epiphyseal regions of long bones. Unlike endochondral bone formation where the cartilage cells perish, the preosseous corpuscles of the antlers appear to become directly converted into osteocytes.

Throughout their entire period of growth, the antlers are supplied with blood by the arteries of the velvet (Waldo, Wislocki and Fawcett, 1949). Venous drainage occurs at first internally through the pedicle (April, May), but by July this pathway has been completely supplanted by a series of efferent veins which are located in the velvet. Recurrent arterioles at the growing tips of the antlers connect the arteries of the velvet with the capillaries and venous channels of the antler shaft. A ligature tied around the antler base in mid-June produced no permanent interference with either the blood supply or growth, whereas ligation in July caused cessation of growth and death of the antler. These results indicate that in June there are enough internal vascular channels to by-pass the blood around the site of the ligature, whereas in July such internal channels are no longer available. Natural shedding of the velvet appears to be associated with ossification of the peripheral zone of the antler shaft, a process which restricts the venous return from the interior of the antler and obstructs the circulation.

With respect to the mode of growth of antlers, in recent interesting experiments two fallow deer were injected with radioactive phosphorus (Bernhard, Brubacher, Hediger and Bruhin, 1953). In a stag with growing antlers, a radiogram showed a gradient of phosphorus deposition which was

maximal in the tines and declined toward the antler base. Another deer with mature, dead antlers which had shed the velvet, was given radioactive phosphorus. The radiogram showed no activity whatsoever in the antler, but some deposition of phosphorus in the pedicle, which ceased abruptly at the line of union with the antler. Alkaline phosphatase activity in Virginia deer as measured biochemically, shows a similar gradient from the antler tips to base (Aub, 1940).

The antlers are well innervated by branches of the trigeminal nerves (Wislocki and Singer, 1946). Numerous small nerves, located in the vascular layer of the velvet, extend outward to the growing antler tips. The dermis of the antlers is supplied with sensory endings for touch or pain. One antler was denervated experimentally in each of two deer just as growth had started. The denervated antlers grew, but became somewhat dwarfed and deformed compared with the normal antlers; they lost their velvet in normal fashion and were ultimately shed. We attributed the somewhat irregular and retarded growth of the denervated antlers to traumatic injury attendant upon loss of their sensory receptors rather than to a loss of a direct trophic influence upon growth.

The nerves supplying the antlers are probably collaterals which grow out from fibres normally innervating the antler pedicles. The fibres supplying the antlers are destroyed annually when the velvet is lost, but they regenerate seven months later upon renewal of the antlers. In the largest species of deer (elk, caribou), the rate of growth of the nerve fibres must exceed half an inch a day, establishing a record for the rate of growth of nerves.

More recent observations indicate that, after the velvet is shed, the proximal portions of the nerve fascicles remain dormant over winter in the skin of the pedicles until they regenerate in the following year (Wislocki and Waldo, 1953). In the dermis, close to the distal edge of the pedicle during the period of dormancy, sizable nerve fascicles are encountered, each of which is enclosed in a heavy sheath of epineurium. The suggestion is offered that these heavy sheaths protect and

shelter the nerve fascicles during the long quiescent period. The protection afforded explains, perhaps, why neuromas are not formed during the extensive period of latency.

Besides the annual cycle of the antlers, male deer exhibit seasonal changes in their gonads and accessory reproductive organs. Thus Virginia deer show seasonal differences in the size and histological picture of the testes, epididymides and seminal vesicles (Wislocki, 1943, 1949). Spermatogenesis begins early in July, becomes maximal in October, declines in December and January and is in complete abeyance from then until July. Similarly, the interstitial cells of the testes are more active in the fall than in the spring, as gauged by various cytological reactions for lipids. The seminal vesicles also reach a peak of seasonal activity in the fall as judged by size and secretory activity. The annual growth cycle of the antlers is controlled by endocrine factors. The effects of castration, reported by numerous previous investigators upon various species of deer, have shown quite conclusively that (1) castration in the first eight months of life results in complete suppression of antler growth, (2) castration after the appearance of the first set of antlers, if they are in the velvet, results in the permanent retention of the velvet and failure of the antlers to be shed, and (3) castration in the presence of antlers which have lost their velvet, results in immediate shedding of the antlers and their subsequent renewal and permanent retention in the velvet. These results rest upon the observations and experiments of Caton (1877), Nitsche (1898), Rörig (1899, 1907), Tandler (1910), Tandler and Grosz (1913) and Zawadowsky (1926), to mention a few.

The present investigator and his associates have confirmed these previous results and have extended the endocrine analysis of the factors controlling antler growth by the administration of testosterone to a series of Virginia deer under various experimental conditions (Wislocki, Aub and Waldo, 1947; Aub, Wislocki and Waldo, 1950; Waldo and Wislocki, 1951). An airgun was devised with which slugs of testosterone propionate and other similar materials could be shot into deer.

The following results were obtained:

(1) Administration of testosterone to bucks which had never had antlers, as a result of castration as fawns, induced antler growth.

(2) Administration of testosterone to bucks, which had been castrated as yearlings or later and which bore permanent antlers in the velvet, induced prompt shedding of the velvet and subsequent shedding of the antlers (tendency to restore the antler cycle).

(3a) Administration of testosterone to normal bucks during the period of antler growth induced shedding of the velvet.

(3b) Its administration to normal bucks, subsequent to the shedding of the velvet, inhibited the normal casting off of the antlers and led to their retention for many months.

(4) Administration of testosterone to ovariectomized female deer caused the growth of well-developed antlers (masculinization).

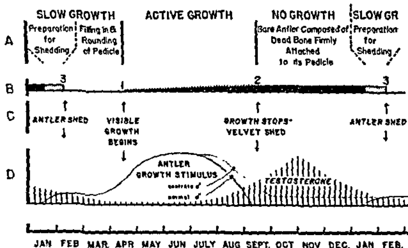
The information gained about the seasonal changes in the antlers and gonads of Virginia deer, combined with the observed effects of castration and administration of testosterone upon antler growth, led to the conclusion that the phases of the seasonal antler cycle in Virginia deer are controlled, on the one hand, by testosterone and, on the other hand, by an "antler-growth stimulus", the latter probably of anterior pituitary origin (Wislocki, Aub and Waldo, 1947; Waldo and Wislocki, 1951). The effects of castration of adult deer indicate the existence of a non-gonadal (hypophyseal) factor responsible for antler growth and of a testicular factor mainly responsible for the secondary hardening (internal reorganization) of the antlers and loss of the velvet. Mating takes place in the autumn, during a time when the testosterone level is high and the antlers consist of bare, dead bone. Afterwards the testosterone level declines, whereas the antler-growth stimulus reappears. In the opinion of these investigators (Waldo and Wislocki, 1951; Wislocki and Waldo, 1953), based upon *histological studies and observations on living* deer, shedding of the antlers is due to the reappearance of

the growth stimulus. Thus, growth rather than death of the tissues at the antler-pedicle junction represents the major factor causing the antlers to be shed. The observed proliferation of fibrocellular connective tissue at the antler-pedicle junction which accompanies the resorption of the osseous connections appears to be primarily responsible for the separation of the antlers from their pedicles. Furthermore, the hypothesis was advanced that the proliferation of the dermis surrounding the antler base exerts an upward thrust upon the underside of the flange-like osseous burr, causing the live tissues in the pedicle to disengage from the dead antler, whereupon minor trauma and mere weight of the antlers hasten their final separation. In a less well-documented study, Gruber (1937) advanced a somewhat similar concept of the process of antler-shedding in the roe deer (*Capreolus capreolus*) and the European stag (*Cervus elaphus*); nevertheless, Bruhin, as late as 1953, still adheres to the older view that shedding occurs during a period of quiescence or latency.

The relation of the phases of the antler cycle to seasonal changes in hormone levels in Virginia deer, as elucidated by the studies cited, are illustrated diagrammatically in Fig. 1.

In 1943, Wislocki pointed out that the growth phase of the antler cycle is initiated and completed in the spring and summer during a period of increasing and maximal daylight, whereas the shedding of the velvet and rutting (increasing gonadal activity) occur in the autumn during a period of diminishing daylight. Experiments were planned at that time to test the possible rôle of light in regulating the antler cycle, but these were subsequently never carried out. Meanwhile, a single experiment on a red deer (*Cervus elaphus*) has been reported by Jaczewski (1952). A stag in which antler growth commenced early in March, was confined daily, beginning on April 1, in a dark stall, from 4:00 p.m. to 8:00 a.m. On June 11, while still confined, the velvet was shed and the stag became aggressive. He was thereupon released and placed out of doors, and on July 12, shed his antlers. New antlers began immediately to grow and by mid-August had developed

two points. Although Jaczewski placed a different interpretation on this sequence of events, the present writer suggests that the reduced daylight from April 1 until June 11, caused both premature shedding of the velvet and rutting, events that would normally have occurred with diminishing daylight



in the late summer and autumn. Moreover, upon return of the animal to full daylight, growth was again stimulated, with loss of the antlers (July 12) and the immediate appearance of a new set of antlers. The complete sequence of events in this stag indicates the stimulating effect of withdrawal of light

upon differentiation of the antlers and gonadal function (pre-mature shedding of the velvet and rut) and the growth-stimulating effect of increase of light by the subsequent resumption of antler growth.

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DISCUSSION

Bourlière: Prof. Wislocki, what is the influence of the age of the animal on the antler cycle? Is the cycle modified in any way in the very old animals?

Wislocki: Senescent changes do occur in deer antlers as revealed by observations on the antlers of old deer and, particularly, by several series of shed antlers which have been collected annually from captive deer. These series reveal a progressive annual increase in size and weight of the antlers up to 8 or 9 years of age, after which there is a rapid

diminution in antler size. A picture of such a series is presented by H. E. Anthony (1929). In view of the concept I have outlined of the control of the antler cycle, I attribute the decline of the antlers in senescent deer to a weakening of the hormonal regulation by the gonads and anterior pituitary.

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removal of the gonads of adult deer. On the other hand, the diminution in their size points to decline in the pituitary factor.

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induced the growth of antlers of incredible size (Vogt, 1937).

Deer ordinarily consume grasses, leaves, shrubs, lichen, berries and other plants from which they usually obtain sufficient minerals. If their needs are not fully met, they may adopt peculiar habits. Murie (1935)

[illegible]

in Scotland, their jaws do not fit and there may be complete malocclusion. When the grass-growing season starts again the tooth again becomes held in the alveolus.

[illegible]

accompanied by local decalcification, to a hormonal "antler-growth" stimulus, probably of pituitary origin. Thus, the shedding of the antlers does not appear to be a regressive phenomenon dependent solely on *poor nutrition*, as would seem to be true of the changes in the teeth of pregnant ewes. However, in connection with pregnancy, it should be

[illegible]

changes in calcium and phosphorus should have occurred during the course of gestation.

In this connection, reference to hermaphrodite deer may be of interest. Hermaphrodites are very prevalent in *Capreolus*, the European roe deer (Rörig, 1899; 1907) and in *Odocoileus*, the white-tail and mule deer of North America (Wislocki, 1954; 1956). Besides occasional true hermaphrodites and pseudohermaphrodites, which invariably possess fairly well-developed antlers associated with minute, atrophic, intra-abdominal testes or ovotestes, there is a relatively large number of deer with normal female genital tracts, which have nothing abnormal about them except that they possess small, deformed antlers which are often permanently in velvet. These animals usually appear to have been pregnant and they are often lactating. I should infer from this that, during the course of pregnancy, either the ovaries and/or the adrenals had produced ketosteroids capable of stimulating the formation of rudimentary antlers. The possibilities that such antlers may be induced by the activity of progesterone or an androgen have been discussed (Wislocki, 1954, with literature).

Strauss: Prof. Wislocki, you said that the antler bone is softer than the normal bone, does it depend upon the phosphorus content of the antler bone?

Wislocki: It is well known to naturalists that the antlers of deer are

capable of constriction so as nearly to obliterate the lumen. The largest arteries appear to have a broad inner, circular coat and an outer,

fallow buck (*Dama dama*). According to him, sebaceous and apocrine

glands in the velvet were innervated by both myelinated and unmyelinated fibres. Nerve ends were also abundantly demonstrable in the corium, epidermis and on hair follicles. The arteries received medullated and unmedullated fibres with terminations localized in the adventitial and medial coats. Previously, I had assumed that the arteries of the velvet, because of their similarity in structure to umbilical arteries, might be devoid of any innervation.

We also denervated an antler in each of two white-tail deer (Wislocki and Singer, 1946), in late spring, and followed the subsequent antler

to repeated bruises and several fractures which they sustained because of the loss of sensation in the velvet.

Boyd: There were no superior cervical sympathectomies—chronic ones?

Matthews: One or two points: first of all, if the initiating of the growth

hormones.

Dempsey: Could it be the pituitary or could it be the end-organ that is triggered?

Wislocki: Well, there is always the possibility of the end-organ. We have considered that but we have so little evidence that they ever got the direct effect on the end-organ.

Zuckerman: Is it possible that you can get other effects in the end-

that?

Matthews: Yes, that is so but is it a very close observation? The fact is that the animal has a deformity in the same place every year, but it is merely guesswork to say that it was caused in the first instance by damaging.

Zuckerman: That is certainly true.

Matthews: Of course, there is another wonderful yarn that goes around among deerstalkers and that is, if an animal gets accidentally unilaterally castrated the horn on the opposite side is always deformed or even absent.

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AGEING OF THE AXILLARY APOCRINE SWEAT GLANDS IN THE HUMAN FEMALE

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THIS work is based on a large number of biopsy specimens collected with a high-speed rotary biopsy punch, 5 or 10 mm. in diameter, from the axillae of normal volunteer subjects. For the study of normal adult glands, specimens were obtained from women 21 to 36 years old; two biopsy specimens were excised at weekly intervals for four weeks from each of the subjects. Fluctuations, if any occurred, in the morphology of the glands during the menstrual cycle could thus be followed. Specimens were taken at monthly intervals from several pregnant women and for two months after parturition. Ageing changes were studied in a series of specimens from subjects 41 to 78 years of age. Fewer but comparable specimens from men were also studied.

Apocrine sweat glands occur in most mammals. In the Primates, the lemurs and the Platyrrhines have only apocrine glands, whereas the Catarrhines have both eccrine and apocrine sweat glands. In the anthropoid apes, the chimpanzee has more eccrine than apocrine glands, but the orang has more apocrine glands (Schiefferdecker, 1922). In the human body apocrine sweat glands are found in the axilla, the mons pubis, the external auditory meatus, the circum-anal area, the areola and nipple of the breast and the labia minora of the female, and in the prepuce and scrotum of the male. Some glands may be found on the face and around the umbilicus. Negroes have more apocrine glands than do Caucasians, and the glands are more numerous in the female than in the male of both races (Homma, 1926). According to Schiefferdecker (1922), the human races can be segregated on the basis of

abundance of apocrine glands; Europeans, having the fewest apocrine glands, belong to the highest order, but the Australian Negroes, with the most glands, belong to the lowest. Actually, the frequency and the distribution of apocrine glands have much the same variety in all races (Woollard, 1930).

Apocrine axillary glands develop in the fifth foetal month as adventitious buds from hair follicles. At the end of foetal life they open into the funnel-shaped depression of the pilosebaceous canal; they are incompletely formed at birth and their characteristic properties develop slowly. Growth, final development and activity are closely related to sexual development and maturity. The glands attain full development at puberty, and they are said to undergo involution in senescence (Ito, Tsuchiya and Iwashige, 1951).

Axillary apocrine glands are compactly coiled, tubular glands (Horn, 1935; Sperling, 1935), with adjacent loops joined by shunts or terminating in blind sacs. The deepest portion of the secretory coils extends into the subcutaneous fat. The single, or, rarely, doubled, duct of the axillary gland, imbedded in the loose connective tissue around the hair follicle, is perfectly straight. It runs near and parallel to the hair follicle and opens inside the pilosebaceous orifice. When ducts open directly onto the surface, the associated hair follicle may have degenerated.

The secretory epithelium is composed of irregularly columnar cells (Fig. 1), with the terminal portion often elongated *and projecting into the lumen. The free border of these cells* terminates in very fine cytoplasmic processes which give the surface of the cells the appearance of a cuticular or brush border. Occasionally, segments of individual tubules, or entire tubules, are lined by simple cuboidal epithelium. When the tubules are excessively dilated, the epithelium is reduced to cells so flat that they resemble endothelial cells. The epithelial cells rest upon a mesh of myoepithelial cells aligned roughly parallel to the axis of the tubule. Outside of the myoepithelial cells is a thick, hyalin basement membrane.

In order to understand senile changes it is necessary to

describe first the histological features in normal adult glands. In discussing the ageing processes only a few of the more striking morphological features will be considered.

The Glands of Young Adults

The axilla contains rows of coiled apocrine glands, each associated with an axillary hair follicle. In histological sections each gland appears as a large nest of tubules cut at various planes (Figs. 1, 3). Connective tissue trabeculae separate adjacent nests; the connective tissue around the gland itself is very delicate. Although every section within each nest is a segment of the same coiled gland, the secretory epithelium of the different segments may be extremely different. In some segments, the epithelial cells are very tall; in others they may be low cuboidal. Some tubules have a small lumen, others have a wide one. Pigment is abundant in the cells of some segments and nearly absent in those of others. These differences seem to record the differences in the state of activity of the various segments. Particularly in the axilla of women over 30, one or more segments may be distended and the epithelium reduced to squamous cells (Figs. 2, 3). In contrast to the lumen of normal tubules, which contains a clear colloid that does not stain with either basic or acid dyes, the lumen of dilated tubules contains a flocculent fluid which stains with basic dyes and often stains a strong metachromatic colour with toluidine blue. The secretory epithelial cells of axillary glands usually contain pigmented granules, but the amount of pigment varies from one individual to another. All of the biopsy specimens removed from the same individual at weekly or monthly intervals show the same characteristic amount of pigment.

In different individuals the pigment granules may be numerous or scant, barely visible or as large as the nucleus. Pigmented granules are aggregated around a clear juxtanuclear region; the terminal cytoplasmic extension of each cell is always free of granules. Some cells may be full of pigment,

Pigment may be found in the tall cells or in the flat ones, and it may be absent from either of them. Neither type of pigment is affected by lipid solvents and both are relatively intact even in paraffin sections. Neither pigment is argyrophilic. The larger, brown granules stain with basic dyes; the small yellow ones are acidophilic. When stained with Altmann's aniline acid fuchsin-methyl green, the small granules, like the mitochondria, are fuchsinophilic; the medium-sized granules stain green, and the large ones remain unstained.

The larger pigmented granules are autofluorescent and emit a yellow to orange light, even in paraffin sections (Bommer, 1929; Montagna, Chase and Lobitz, 1953). The small yellow pigment granules are either nonfluorescent or emit a pale yellow light of very low intensity. The secretion fluid in the lumen of the tubules emits a very pale autofluorescence.

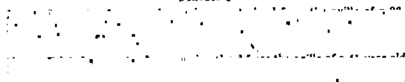
The dark pigment contains some lipid and can usually be coloured with sudan black. The sudanophilia of these granules can be demonstrated in paraffin sections (Fig. 7). The yellow pigment is not sudanophilic. Many of the larger pigment masses, but not the small yellow pigment granules, are acid-fast and can be stained with carbolfuchsin and differentiated with hydrochloric acid. The larger pigment granules are mildly positive with the plasmal reaction, indicating the presence of compounds containing aldehydes, ketones, acetals or compounds containing unsaturated groups (Bunting, Wislocki and Dempsey, 1948).

The large pigment granules are reactive to the periodic acid-Schiff method. The reaction is not diminished by previous treatment with diastase or saliva (Fig. 5).

The epithelial cells of axillary glands characteristically contain ionic iron (Bunting, 1948; Bunting, Wislocki and Dempsey, 1948; Cavazzana, 1947; Homma, 1925; Homma, 1926; Iwashige, 1951; Montagna, Chase and Lobitz, 1953; Zorzoli, 1952). Homma reports that the glands in 70 per cent of the specimens obtained from cadavers and from surgery contained iron, and Manca (1934) found it in 26 out of 27 specimens. Some specimens abound in iron, but others may have

traces or none at all. Iron is said to be normally more abundant in specimens from middle-aged subjects. The distribution of iron is erratic; adjacent tubules in the same specimen or separate coils of the same glandular unit may show different amounts of iron. Some cells may abound in iron whereas morphologically identical cells may possess none. The presence or absence of iron is an individual characteristic. Iron is nearly always in the form of small granules; large iron granules are very rare (Bunting, Wislocki and Dempsey, 1948; Homma, 1925; Homma, 1926). Iron may be present in the tall, apparently secretory cells, as well as in the low cuboidal ones. Regardless of the amount of iron in the epithelium, the residual secretion in the lumen of the tubules contains none (Montagna, Chase and Lobitz, 1953; Zorzoli, 1950), unless the epithelium is collapsing and undergoing obvious degenerative changes. Iron is found as a part of, or together with, the small yellow pigment granules; the large dark-brown granules rarely contain it. When they do, only a thin film at the periphery is reactive to the iron tests, and the brown pigment shows through unchanged. The amount of pigment in the cells is no indication of the presence of iron, but iron is found only in the small yellow granules and only if the large brown ones are also present in the same cells. This has led Manca (1934) to call the pigment which contains iron, haemosiderin, the one which does not, wear-and-tear pigment. However, the yellow pigment cannot be called haemosiderin since it may or may not contain iron. Perhaps an iron-containing substance may or may not be associated with the yellow pigment (Zorzoli, 1950). It is possible that whether or not iron is present, the yellow

PLATE I



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PLATE II.

Fig. 4. Low power field of the dilated tubules of the glands from the axilla of a 74-year-old woman. The epithelium in nearly every tubule was cuboidal or low columnar.

Fig. 5. Secretory cells from the glands of a 26-year-old woman. Stained with the periodic acid-Schiff method showing Schiff-positive granules.

Fig. 6. Perfectly normal cells from a gland in the axilla of a 61-year-old woman. Stained with the periodic acid-Schiff method. Compare with Fig. 5.







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pigment granules could develop into the brown ones. When they contain iron, the yellow pigment granules gradually lose iron, or perhaps the inorganic iron is gradually transformed into organic iron. However, there might be two yellow pigments, only one of which contains iron. Ionic iron is never found in the terminal cytoplasm of the cells.

Not all axillary apocrine cells contain pigmented granules. In the glands of some individuals the secretory cells possess *only* traces of pigmented and *lipoidal granules. In their* places, the supranuclear cytoplasm has usually chromophobic and occasionally slightly basophilic secretion spherules. In some subjects these spherules are the only secretory elements present; in others they may be admixed with pigmented ones. *With Heidenhain's haematoxylin the "chromophobic"* spherules stain a clear blue-black. They are Schiff-reactive but resist digestion with diastase or saliva.

The base of each apocrine cell is usually stippled with numerous fine basophilic granules, which often gives these cells a longitudinal striation. The cytoplasm lateral to and above the nucleus is weakly basophilic. In each cell a spherical region above the nucleus, which corresponds to the negative *image of the Golgi* apparatus, *is free of basophilic granules.* The apical and terminal cytoplasm of apocrine cells is almost

PLATE III

FIG. 7. Sudanophilic pigment granules in an axillary gland from a 28-year-old woman. *Paraffin section coloured* with Sudan black.

FIG. 8. Normal distribution of sudanophilic pigment granules *in an axillary* gland from a 78-year-old woman. Compare with Fig. 7. Paraffin section coloured with Sudan black.

FIG. 9. Iron in the axillary gland of a 78-year-old woman. *The large pigmented granules* are unreactive to this test. Section treated with the Prussian blue method of Gomori.

FIG. 10. Epithelial cells from a dilated gland from the axilla of a [illegible] woman. The lumen is to the left of the cells. The granules in the cyto[illegible] stained metachromatically with toluidine blue.

FIG. 11. Flattened epithelial cells from an axillary gland [illegible] woman. The cytoplasm contains glycogen granules. The floccul[illegible] the lumen is Schiff-reactive. Stained with the periodic acid [illegible]

FIG. 12. Intensely stained colloid in an axillary gland [illegible] woman. Section treated with the periodic acid [illegible]

chromophobic. The basophilic granules, as well as the nucleoli, are no longer stainable with basic dyes after the sections have been incubated in ribonuclease, and they must therefore contain ribonucleic acids. The intensity of basophilic staining in the cytoplasm of secretory cells is inversely proportional to the number of recognizable secretion granules present. Thus, nucleic acid may be involved in the synthesis of the secretion granules, pigmented or chromophobic.

Normal axillary apocrine glands contain no glycogen (Montagna, Chase and Lobitz, 1953). However, Schiff-reactive substances other than glycogen are numerous in the apocrine cells and have already been described (Fig. 5).

Spindle-shaped myoepithelial cells are couched between the single layer of columnar or cuboidal cells and the basement membrane. These cells are 5 to 10 μ in the widest diameter and 50 to 100 μ in length. In transverse sections of tubules they appear as triangular cells between the bases of the secretory cells. The axes of the cells are oriented roughly parallel to that of the tubule. Myoepithelial cells are best developed in those tubules which are lined with the tallest epithelium. When tubules are dilated and the epithelial cells are flat and elongated, myoepithelial cells are very delicate or impossible to demonstrate.

The Ageing Glands

In the glands of women around 30 years of age, a few segments of tubules become dilated and the secretory epithelium is reduced to cuboidal or squamous cells (Fig. 2). This is actually the onset of ageing changes. Between the ages of 34 and 44 there is a very gradual increase in the number of dilated segments in the entire axilla, and in a group of women 40 to 44 years old, entire glandular units have a flattened epithelium with or without an accompanying dilatation of the tubules (Fig. 3). The dilated tubules are lined with squamous cells. Although these atrophic changes can be found in nearly all specimens from the late thirties to the late forties, or up to

the period just before menopause, they are not numerous. The majority of the glands are normal and their secretory cells are tall columnar. In specimens from progressively older women more tubules are lined with a flat epithelium and a larger number of tubules is dilated. The lumen of the tubules lined with the atrophied epithelium contains a flocculent residue, in contrast to the lumen of normal tubules, the content of which is always clear. The most marked ageing changes in the axillary organ occur in the early fifties. At this period many entire glandular systems become atrophied. Atrophy and dilatation of the glands progresses very slowly from here on. We have not found a single specimen, regardless of age, in which there were not at least a few apparently normal and functional glands. This point is subject to great individual variation. In some individuals the glands become ineffective much more rapidly than in others. Many of the tubules in the axilla of a 78-year-old woman appeared normal.

The assumption that the epithelium of these glands is normal is drawn from several criteria. The epithelial cells, although subject to variations, are usually tall columnar, with some cytoplasmic extensions projecting from their apices. The cells contain variable amounts of brown and yellow pigment granules. The small yellow granules often contain iron (Fig. 9), and the brown granules are usually sudanophilic (Fig. 8), acid fast, autofluorescent, and Schiff-reactive (Fig. 6).

In contrast with the glands which remain intact, the epithelium of the glands which show atrophic changes has lost most of the characteristic features found in apocrine cells. Morphologically, the cells have lost the resemblance to secretory cells. They may be so flat as to resemble endothelial cells (Fig. 10). They rarely contain granules and pigment, lipid or iron. In contrast with normal glands, which never contain it, glycogen may be found in the epithelial cells (Fig. 11) and in the myoepithelial cells of the atrophied tubules. In addition to these granules of glycogen, the cells may also contain saliva-resistant, Schiff-reactive granules. These same granules also stain metachromatically with toluidine blue (Fig. 10). This

combined staining property is identical with that of mucus. *Also, the curdled luminal content of these glands stains* like the granules just described. This, too, is like mucus, and is strikingly different from the serous content of normal glands which is clear and nearly achromic.

As soon as the epithelial cells become flattened, the *myoepithelial cells become very thin and inconspicuous.* In the tubules lined by squamous cells myoepithelial cells cannot be demonstrated. The presence of conspicuous myoepithelial cells, then, is a property of the functional glands.

The duct is the most resistant structure of the axillary gland *and it is never changed morphologically. All of its features* are like those of normal mature glands, even in those subjects in which the glandular tubule is reduced to a series of thin-walled cysts. This point is of some interest. Perhaps in spite of the changes which the glands suffer with ageing, they still *retain some secretory activity, although the type of secretion* is considerably altered.

Mitotic figures are not infrequent in the glandular epithelium of aged subjects. It must be recalled that mitosis is not common even in the glands of young women. In the glands of women 50 years of age or older mitotic figures may be found even in the flattened epithelial cells. If these are self-propagating cells, then the changes that they have undergone are not degenerative.

Axillary glands are inactive in children, become functional at puberty and remain active throughout the sexual life. In old age axillary odour seems to disappear, indicating a decrease and final cessation of apocrine secretion. In women the glands are believed to undergo periods of greater or lesser activity correlated with the menstrual cycle and with pregnancy.

Authors generally agree that the glands are small and inactive during the intermenstruum and become swollen and active during the premenstruum and during the menstruum (Loeschcke, 1925; Schaffer, 1926). Cavazzana (1947) believes that during the premenstruum and menstruum the glands are actually larger than during the intermenstruum; their

lumen is dilated, and the secretory cells average taller. The myoepithelial cells are believed to be swollen and farther apart during the menstruum. During pregnancy the changes are like those which occur during the menstruum but they are much more pronounced (Talke, 1903; Waelsch, 1912). Contrary to all others a few authors (Cornbleet, 1952; Loeschcke, 1925; Richter, 1932) believe that during pregnancy the axillary glands are in a resting state or that their function is depressed. Although these concepts are accepted unquestioningly, evidence to the contrary deserves some scrutiny (Klaar, 1926).

Unlike the other authors, all of whom studied apocrine glands in autopsy material, Klaar (1926) obtained biopsy specimens from the same women at weekly intervals during the menstrual cycle. He found in normal women no changes in the axillary organ which correspond to the cyclic menstrual changes, and concluded that there is no correlation between functional activity and the diameter of the gland, and that the differences in the height of the secretory epithelial cells and their content of iron in no way corresponds to the menstrual cycle. During pregnancy and in puerperium the glands exhibit individual range of variation similar to that found in non-pregnant women. In women after menopause, the glandular epithelium exhibited a wide range of variations; they all had some characteristic dilated or "cystic" tubules lined by flattened epithelial cells and surrounded by a thick connective tissue capsule. Normal glands, however, could be found alongside of the cystic tubules even fifteen years after the onset of the menopause. In castrated women a general slow regression of the apocrine glands is similar to that seen after the menopause.

Our observations are in complete agreement with those of Klaar. We have been unable to correlate any changes in the axillary glands with the menstrual cycle. Similarly, no changes have been found in biopsy specimens removed at monthly intervals from pregnant women. Thus, there do not appear to be definite cyclic changes in the axillary apocrine

glands which parallel those of the gonads. The contradictions between these reports and those of others may be the result of the use of improper and insufficient materials by others. It seems possible that most authors have lacked an appreciation of the full range of variations which occur in the glands even in the same individual. Although it is customary to believe that axillary apocrine glands have a cyclic secretory activity, we have not been able to detect cyclic changes in their morphology.

Usually everyone assumes that the glands become functional at the onset of puberty. It cannot be denied that gonadal maturation largely coincides with the onset of strong axillary gland activity. Yet the glands of infants are clearly recognizable and larger than the eccrine glands. The glands of female children or juveniles which are not yet menstruating are often large and functional (Klaar, 1926). The glands, then, do not respond specifically to ovarian hormonal stimulation; this is also reflected in the ageing processes. Although castration and menopause bring on some ageing changes quickly, many entire glandular units remain intact and apparently normal for years after these events. In some of the glandular units, regressive or ageing changes take place in some coils of the tubule while others remain normal. Thus, ovarian hormones play an obvious rôle in initiating their maturation and maintaining in part the functional state of the axillary apocrine glands, but other hormones, perhaps from the adrenal cortex, are probably much more closely implicated in this. What makes this assumption more probable is the fact that in ageing men the glands show far less striking regressive changes than those of women.

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DISCUSSION

framework. I do not know whether his serial biopsy technique would reveal such a process if it occurred; but if there were a progressive deterioration in the replacement power of these glands, that might at least account for the fall off in the number of functional units in the population.

Montagna: It is evident from our material that some glands become aged more rapidly than others. I do not know whether or not the same

evidence of that?

Montagna: This is a controversial point. We collected specimens once a month from eight women from about the third month of pregnancy through parturition and lactation, until their menstrual cycle had been restored. We found nothing in the axillary glands that we could say correlates with these changes.

The only other person who has collected repeated biopsy specimens from the same subjects over the menstrual cycle, is Klaar. We have confirmed his work and we are the only ones who agree with him. We are in disagreement with all others, who have relied entirely upon autopsy material.

Strauss: Prof. Montagna's magnificent paper is a very important morphological contribution to the understanding of the life curve of the human sex appeal! He mentioned very briefly, but very importantly for the anatomist, that he does not like to call the glands "apocrine glands". I would like to ask him why not?

Montagna: This is a matter of definition. In apocrine glands the head-portion of the secretory cells should pinch off and disintegrate to form the secretion. Whereas this takes place *in some* cells, most cells secrete by giving off little globules of secretion at the brush-border. I think that that is the way most of the secretion takes place.

Strauss: You would call them eccrine glands?

Montagna: No! Since one cannot combat terminology by creating new terms I stick with the old terms!

Amoroso: An essentially similar view has been propounded by Richardson in respect of the secretion of milk.

Huggett: I am interested in certain points. I gather that you have not been able to test these more mysterious portions on animals—you might get animal material. I was wondering what is the distribution of these glands in animals and whether you might not be able to satisfy —shall I say that sex appeal—by getting animals who are prepared to supply their skin?

Montagna: Although we have done a great deal of work on other mammalian glands, they are not nearly so interesting. Most tubular glands in other mammals are fairly dull; they secrete very deliberately, with the exception of those of the horse and the pig which secrete fairly profusely. The inguinal glands of the rabbit are large but they secrete only traces of a musky smelling substance.

Huggett: This is a species story?

Montagna: I am afraid it is.

Huggett: And you have not got an animal species you can use as an experimental subject?

Montagna: No. Even among the Primates, the lemurs and the Platyrrhines have only apocrine, whereas the Catarrhines have both eccrine and apocrine. The chimpanzee has more eccrine [illegible] than apocrine, and the orang has very few [illegible]

Huggett: To what [illegible] You spoke of the male [illegible] do not know what the word "*de*[illegible] *that context.*

Montagna: The change is very much slower. I mentioned that in

women at menopause there seems to be a precipitation of these changes whereas there is no event in the male which is comparable to that. The changes are all very slow, gradual ones.

Huggett: Well, you mentioned that in the girl the apocrine gland begins to show its differentiation about 8 or 9 years of age; does anything similar happen at that date in the boy?

Montagna: Not as pronounced. These observations were all made with our noses! Most parents, and particularly the mothers, probably know this. This is an empirical observation and I have nothing to substantiate it. I was talking about this with Dr. Rothman and he made the delightful comment that dermatologists were not aware of this.

Zuckerman: Among Primates you looked at, did you examine the pigtailed macaque?

The rapid formation of amides in ageing tissues may represent a gradual alteration in the balance between synthesis and breakdown.

There is now much evidence that the proteins and other nitrogenous constituents of plant tissues are maintained in a continuous dynamic equilibrium. The operation of independently controlled processes of synthesis and breakdown was earlier suggested by Mothes (1933) and by Gregory and Sen (1937), and the use of isotopic nitrogen (^{15}N) has provided direct evidence of the dynamic state of leaf proteins. The experiments of Hevesy and others (1940) and of Vickery, Pucher, Schoenheimer and Rittenberg (1940) demonstrated the incorporation of the isotope into tissue proteins in considerably greater quantities than could be accounted for by primary synthesis. More decisive evidence has been obtained from the experiments of Chibnall (Chibnall and Wiltshire, 1954) with leaves of runner bean and from our own experiment with barley leaves. It has been shown that, even under conditions in which a net loss of protein occurs in detached and senescent leaves, there is appreciable synthetic activity indicated by the active incorporation of ^{15}N into the tissue protein.

Some of the results of an experiment, in which a dilute solution of ammonium phosphate, containing about 30 atom per cent excess of ^{15}N, was supplied to the cut surface of detached barley leaves, are summarized in Table I. The protein separated from the leaves was subjected to group analysis by methods similar to those described by Yemm (1950) and the abundance of ^{15}N determined in each group.

Under the conditions of this experiment a net loss of about 20 per cent of the tissue protein occurred during the period of 24 hours over which the experiment extended. Nevertheless, it is evident from the data of Table I that considerable synthetic activity continued in the leaves; a significant incorporation of isotopic nitrogen was detected in each of the main groups of amino acids into which the protein was analysed. The highest abundance of ^{15}N was observed in the

dicarboxylic amino acids and in the amide N, again emphasizing the high activity of glutamic and aspartic acids and their amides in the protein metabolism of the leaves.

It may be concluded from the evidence outlined above that the protein content of leaves is the result of continuous synthetic and catabolic activities. In ageing tissues the loss of protein is attributable to a decline in synthetic capacity, leading to an adverse balance and the predominance of catabolic changes as yellowing of the leaf proceeds.

Table I

INCORPORATION OF ^{15}N INTO PROTEINS OF BARLEY LEAVES

Protein separated from leaves 24 hours after supplying them with 0·04 M-$NH_4H_2PO_4$ containing 29·3 Atom % excess ^{15}N.

	Atom % Excess ^{15}N
Total N	0 183
Dicarboxylic amino acid	0 210
Basic amino acids	0·080
Other amino acids	0·160
Amide N	0·441

Respiratory Activities of Senescent Leaves

The changes of respiratory activity in yellowing leaves have been recently reviewed by James (1953). Much of the available data with leaves of different species has been obtained with detached leaves often kept for long periods in the dark. As already noted, senescence occurs rapidly under these experimental conditions, but in the present context it is of interest to examine briefly the general relation between the rate of respiration and yellowing of the tissues. The pioneer research of F. F. Blackman (see James, 1953), carried out with leaves of Cherry Laurel and Tropaeolum, showed that during yellowing of mature leaves a marked increase in the rate of respiration took place. In leaves of different species

the time scale of these changes varies widely; the rise in CO_2 production and rapid yellowing occurred after about 25 days in Cherry Laurel and after 8–10 days in Tropaeolum leaves. With mature leaves of barley and of grasses the changes are much more rapid and yellowing often occurs 2–3 days after detachment from the plant. Despite these wide differences in the rate of ageing, many of the same features can usually be recognised; during yellowing relatively high or rising rates of respiration are generally observed with detached leaves of different species. More limited data are available concerning the respiration of leaves when they undergo normal senescence while still attached to the plant. However, Arney (1947) in experiments with attached strawberry leaves has shown that an appreciable rise in rate of CO_2 output occurs during yellowing.

A detailed interpretation of the respiratory drifts in ageing leaves will not be attempted here. There is strong evidence that the respiratory mechanisms are considerably modified; with detached leaves of barley and other species, chemical analyses indicates that during yellowing a substantial part of the CO_2 lost from the tissues is formed by the oxidation of the carbon skeletons of amino acids. Godwin and Bishop (1927) found that a loss of relatively stable glycosides and probably polysaccharides took place during the senescence of Cherry Laurel leaves. High rates of respiration seem, therefore, to be closely related to the breakdown of proteins and other complex constituents in senescent leaves. Soluble metabolites, such as amino acids formed in this way, may be important respiratory substrates which sustain the cellular oxidations associated with the rapid release of CO_2 from ageing tissues.

In addition to leaves, some other senescent plant organs show an acceleration of respiration during ageing. The so-called climacteric rise, observed in apples when they ripen and become yellow (Kidd and West, 1930), has been shown to occur in many other ripening fruits. The metabolic changes which take place in apples have been extensively studied (see, for example, the review by Pearson and Robertson 1954);

the high rates of respiration in the ripening fruits are associated with the breakdown of starch to sugars, although a slight synthesis of protein can be detected during yellowing.

The experimental data briefly considered above indicate that the catabolic processes in senescent plants are commonly associated with high rates of cellular respiration. It is appropriate now to try and distinguish some of the basic changes in cellular metabolism which accompany ageing of plant tissues, and to bring the data into relation with some current views on the regulation of respiration and its coupling with synthetic activities.

The Relation between Cell Respiration and Protein Metabolism in Ageing Tissues

Gregory (1937), Richards (1936) and their collaborators have demonstrated a close connection between respiration and protein metabolism in leaves of barley. In a tentative hypothesis Gregory and Sen (1937) suggested that a continuous protein cycle operates in leaves and that under normal circumstances much of the respiratory CO_2 arises from the oxidation of the carbon skeletons of amino acids. The hypothesis has been further developed by Steward and his collaborators (see, for example, Steward and Thompson, 1954), and, along these lines, the rapid respiration of senescent leaves may be related to the high levels of available substrate, resulting from the breakdown of tissue proteins to amino acids.

There are, however, other cellular mechanisms which may link respiration and protein metabolism and which seem to offer a more comprehensive interpretation of the catabolic changes in ageing leaves. It is now widely recognised that phosphorylation and phosphate carrier systems constitute important mechanisms regulating glycolysis and respiration and coupling them with endergonic synthesis. Experimental work with both animal and plant tissues indicates that the rate of respiration may be strongly influenced by the

availability of free phosphate and/or phosphate acceptors, both of them essential components of the enzymic systems engaged in glycolysis and oxidation. The general importance of phosphorylation in regulating cell respiration in plants has been discussed by Lardy (1952), by Millerd and Bonner (1953) and, with particular reference to protein metabolism, by Folkes and Yemm (1954) and Yemm (1954).

Evidence that the level of phosphorylation may regulate the rate of respiration in plant cells rests mainly upon the action of so-called uncoupling agents, such as dinitrophenols. As reviewed by Simon (1953), these reagents at low concentrations strongly inhibit phosphorylation, whereas enzymatic oxidations are but little affected: under suitable conditions synthetic activities, particularly protein synthesis, are prevented, while a marked increase in cellular respiration generally occurs. Thus treatment of the tissues with nitrophenols and other uncoupling agents produces experimentally changes in metabolism similar to those associated with senescence. Observations such as these have led to the view that ageing in plant tissues is associated with a partial failure of the linkage between phosphorylation and cell oxidations. As a result of this failure, the regulating action on cell respiration is impaired and there is a decline in the synthetic activities whereby the proteins and other complex constituents of the cell are maintained.

Pearson and Robertson (1954) have reviewed some evidence that changes, such as those outlined above, occur during the ripening of apples. They have shown that the relatively low rate of cell respiration during the pre-ripening stages could be substantially increased by 2 : 4-dinitrophenol, but that after the climacteric rise and yellowing similar treatment had little effect. Some further evidence that changes of the phosphorylating mechanisms were associated with ripening was obtained by Biale (1954) from a study of cytoplasmic particles, probably mitochondria, prepared from the avocado fruit. But, as yet, no comparable data have been obtained concerning the metabolism of senescent leaves.

Conclusions

With regard to respiratory activities and the metabolism of proteins in ageing leaves the following conclusions may be drawn from the data:

1. The predominance of catabolic changes in the tissues results, in part, from a decline in synthetic activity, and not solely from an increase in hydrolytic breakdown of complex constituents.

2. High rates of respiration are generally found in yellowing leaves; *much of the carbon dioxide may arise from the* oxidation of the carbon skeletons of amino acids.

3. It is possible that both the high rate of respiration and the loss of synthetic activity result from changes in the phosphorylating mechanisms of the cells.

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DISCUSSION

[illegible]

...the second one: ecological factors and leaf senescence really are extremely important. One of the things which increases the rate of senescence of leaves very markedly in cereal plants, which I have been interested in particularly, is water supply. [illegible] gets much earlier [illegible] under conditions [illegible] other factors—the [illegible]; under conditions [illegible] become senescent [illegible]

Yemm: [illegible]

[illegible] independent.

Huggett: What about leaves on tropical plants in humid climates like Nigeria?

Yemm: Most of these leaves are not deciduous. They have quite a long life and are commonly relatively resistant leaves of the type of Cherry Laurel.

Montagna: The factor of light is also rather important. I made some [illegible] hat I thought to be a very perspicacious observation when

diverted from its normal course and consequently plastid structures and pigments in the cells tend to break down, so that yellowing is here a pathological effect resulting from the diversion of protein metabolism rather than extensive catabolism of protein.

Villee: I would like to congratulate Dr. Yemm on his work; it is very elegant and extremely interesting. I was interested in the comparisons which can be drawn between protein metabolism in animal tissues and plant tissues. I noted from your ^{15}N data that the picture is quite similar in the two, that the highest rate of incorporation was into aspartic and glutamic acids, other than the amide N, and that the basic amino acid lysine had a very low rate, suggesting that, as in animals, lysine probably does not exchange its nitrogen. You stated that glutamine accumulates rapidly, more than can be accounted for by that present in the protein; is that as glutamine or as glutamine plus glutamic acid?

Yemm: As glutamine alone. Practically the whole of the soluble glutamic acid in these tissues is combined as the amide.

Villee: I see. It would suggest a *de novo* synthesis of glutamic acid and the formation of the amine from that.

Yemm: Yes. The deviation of these data depends upon a knowledge of the composition of the leaf proteins. We have separated tissue proteins from the leaf and estimated their average composition in terms of amino acids. We then measured how much protein disappeared over a given period of time and also the amount of glutamine that accumulated. When the two are compared, it is found that glutamine accumulates in much greater quantities than can be accounted for simply by hydrolysis of the proteins. A similar result was obtained for the other common amide of plants, asparagine.

Villee: This, of course, is quite understandable biochemically because of the amination of the corresponding keto acids. Now, I was also interested in the increase in respiration which occurs. Since we know that substituted phenols are accelerators of respiration, I wonder whether the breakdown of pigments such as anthocyanins might in some way release either nitrophenols or chlorophenols which could be a stimulating factor at this time. The coincidence of this with the yellowing of the leaf suggests that perhaps in the yellowing, some of the pigments themselves break down and release physiologically active substances.

Yemm: I have no comment on it. I think that it is difficult to account for its occurrence in different types of leaf, very different in composition and in their anthocyanin pigments; yet the acceleration of respiration seems to be a general phenomenon in senescent leaves and is closely correlated with yellowing and with protein metabolism.

Villee: Have you made any estimates of phosphorus and oxygen ratios to see whether there is, in fact, a change in this ratio which would be the crucial point as to whether phosphorylation has altered later on?

Yemm: I can only say that we have tried it without success. The situation in plants, I think, is here perhaps a little more complicated than in animals. It seems that there are, in all probability, a large

photosynthesis.

Yemm: Yes, but of course all the respiratory data are obtained in the dark. *The two processes are separated in that way. In point of fact,* with barley and some of the other leaves, respiratory quotients have

may come from many different things. And, of course, the oxygen which leaves the plant as CO_2 does not necessarily come in as gaseous oxygen.

Williams: I would like to go into the question of ageing in general. Dr. Yemm seems to be dealing with the ageing of transient tissues but as far as I can gather it seems to be rather for the benefit of the per-

organism.

The second point

viral infections and pathological conditions which leads to the gradual decline in the vigour of the plants. I think that many plants can reproduce themselves vegetatively for a very long time without showing "senescence" in the clone as a whole.

P[illegible]

of [illegible]

I [illegible]

m[illegible]

[illegible]

s[illegible]

words, replace[illegible]

Yemm: Not[illegible] certain syntheses become more obvious, but whether it is a result of a change in the whole balance of metabolism one does not know. The one I was mentioning—glutamine—now this is a synthesis which becomes much more conspicuous in older leaves, but in our view the increase of glutamine may result from an alteration in the balance between synthesis and breakdown of protein as the leaf ages.

Huggett: Are [illegible] yellowing? [illegible] there are [illegible] not seem to be correlated?

Yemm: All [illegible] that [illegible] senesc[illegible] in sy[illegible] capa[illegible] nece[illegible] betw[illegible]

THE PHYSICAL INSTABILITY OF HUMAN RED BLOOD CELLS AND ITS POSSIBLE IMPORTANCE IN THEIR SENESCENCE*

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According to Comfort (1954) in a review of the biological aspects of senescence: "All theories of senescence at the present time must be based on unwarrantable assumptions in the absence of concrete answers to the essential questions of fact". One assumption which seems generally acceptable however is that senescence is in some way connected with "wear and tear", or in physical terms with the natural tendency for disorganization or entropy to increase with the passage of time.

The notion that senescence is due to wear and tear in a mechanistic sense, namely, that living organisms wear out as a result of their continued activity, is probably erroneous but lies behind the widespread belief that the slowing or arrest of metabolism at low temperatures would lead to suspended viability. In recent years a considerable body of information has accumulated on the effects of low temperatures on living cells, and this resulted primarily from the important discovery [illegible]

in the frozen state for periods greater than their normal life-span. It is found, however, that living cells do not necessarily survive for long periods when their temperature is lowered to a level sufficient to reduce metabolism to a negligible value. The progressive loss of red blood cells stored at $-78°$ has been reported by Mollison, Sloviter and Chaplin (1952), and at this

* This paper was presented by Dr. A. S. Parkes on behalf of Dr. Lovelock. —Ed.

temperature ovarian tissue may also fail to survive even one week, although under otherwise similar conditions it survived a year or more at $-190°$ (Parkes and Smith, 1953). The survival of red blood cells at $-20°$ is poor compared with that at $-78°$, although at both temperatures glycolysis has slowed to a point where there is a negligible consumption of glucose during the survival of the cells (Chaplin *et al.*, 1954).

The notion that the cessation of activity would lead to the suspension of vital processes assumes not only that the harmful effects of wear and tear result directly from activity, but also that the cell is stable in a physical sense in the absence of activity.

Nowadays it is more usual to regard living cells as dynamic entities. It is thought that their contents, and possibly also their structures, are maintained at a constant level by a balance between the accretion of synthesis and the losses due to chemical change and to passive diffusion. If a cell, which is in dynamic equilibrium at its normal temperature, is cooled to a temperature where metabolism ceases, or is greatly slowed, it will continue to suffer the loss and disorientation of its components by random molecular movement and by diffusion, for these physical processes are only slightly affected by a fall in temperature. It follows that the life-span of a cell, which is normally in dynamic equilibrium, will be short if it is stored at a temperature where the balance between synthesis and diffusion is no longer on the credit side. It may well be significant that the successful cold storage of living cells has so far only been achieved under conditions where diffusion itself is greatly slowed, namely at very low temperatures, in the presence of viscous substances such as glycerol, or in the dry state.

The concept of the living cell as a steady state system is helpful not only in emphasizing the inadequacies of the purely mechanistic approach to the manifestations of entropy, but also in suggesting the more probable nature of the wear and tear process suffered by the cell, namely, the loss and disorientation of its substance by diffusion. Diffusion is usually regarded

as a relatively slow process, but at cellular dimensions it can proceed with considerable rapidity. This is illustrated by a calculation (Ponder, 1948) showing that 90 per cent of the haemoglobin of a red cell can diffuse in four seconds through a hole occupying only one thirty-thousandth of the cell area.

In comparison with the information available concerning the metabolism of various types of living cell, little is known of the spontaneous diffusion processes to which they are continuously subjected. One exception, however, is the red blood cell; it is well established that its internal contents are not in thermostatic equilibrium with the environment but are maintained at a steady level by active processes. While some of the structural components and the haemoglobin are apparently inert (Muir, Neuberger and Perrone, 1952), it has been shown that the cell lipids are in a condition of rapid turnover both by metabolism (Muir, Perrone and Popjak, 1951; Altman, 1953), and by passive diffusion (Gould, 1951). The integrity of the red cell depends upon the presence of the lipids, and since these substances are free to diffuse away, then to some extent the cell itself can be regarded as a steady state system maintained intact by its metabolic activity.

This information, together with the knowledge that the normal life-span of the red cell is known with considerable precision and the fact that it is particularly convenient for experimental purposes, decided the choice of this cell for the investigation of its physical stability, even though it was realized that the red blood cell is highly specialized and cannot be considered representative of living tissue.

This paper describes some experiments on the diffusion of components from the red cell and its ensuing dissolution. A detailed account of the experimental material and methods is described elsewhere (Lovelock, 1955*a*). The loss of substance was accelerated by suspending the cells in a medium maintained unsaturated with respect to the components of the cell membrane. This was achieved either by repeated washing or by including in the medium a neutral adsorbent substance, namely alumina. A few observations are also included on the

dissolution of red cells during their storage at low temperatures. The progress of the dissolution is described and the structure and state of the cell membrane discussed in the light of the experimental observations. The possible relevance of these observations to the problem of senescence is also discussed.

The dissolution of red cells in the temperature range 0° to +40°

The suspension of human red cells in 0·16 M-NaCl is followed by a loss of lipid components from the cell membrane and to a lesser extent by haemolysis (Lovelock, 1954). The loss of lipids is rapid at first, but after 3 minutes an equilibrium level is reached, and the rate of loss falls to a low value. The equilibrium concentration of lipids does not appear to represent a simple saturation of the medium with these substances,

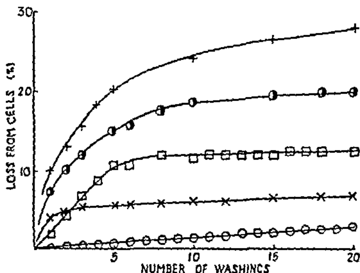


FIG. 1 [illegible] repeated wash[illegible] [illegible]0 min. at 37°, [illegible] [illegible]resh 0·16 [illegible]

+ Li[illegible]; □ [illegible]; ◑ [illegible]holesterol;
× Phospholipid; ○ Haemoglobin.

but depends upon the previous treatment of the cells. It increases with the length of time the cells have been stored *in vitro* and decreases with the number of times they have been washed.

The effect of repeated suspensions in 0·16 M-NaCl is shown in Fig. 1. Even after 20 washes only 6 per cent of the cells have haemolysed, and the proportion haemolysing was constant for each washing. During the first 7 washes, however,

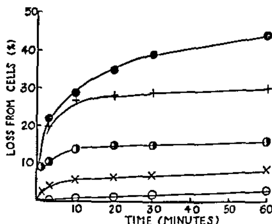


Fig. 2. The rate of removal of components from the red

a considerable amount of the cell membrane was dispersed in the medium and shrinkage of the cells took place. Thereafter the losses of cell components proceeded at a rate equivalent to the loss of cells by haemolysis.

Figs. 2 and 3 show that the effects of exposure to alumina are closely similar to those of repeated washing. The haemolysis of the cells is directly related to the time of exposure and concentration of alumina. The loss of lipids, however, bears a direct relationship to the time and intensity of exposure only

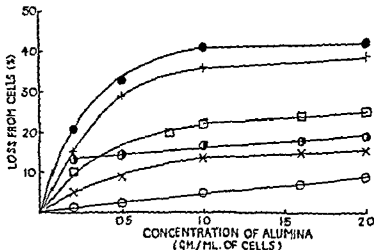


FIG. 3. The removal of components from the red cell by various concentrations of powdered alumina. *Cells* suspended in 0·16 M-NaCl were exposed for 15 min. at 37° to concentrations of alumina from 0·2 g. to 2·0 g. per ml. of packed cells.
● Dry weight of lipids; + Lipoprotein; □ Volume; ◐ Cholesterol; × Phospholipid; ○ Haemoglobin.

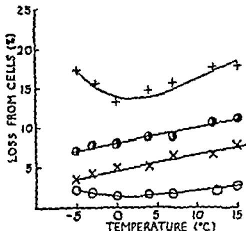


FIG. 4. The removal of [illegible]
various temperatures. [illegible]
to 1·0 g. of alumina pe[illegible]

+ Lipoprotein; ◐ C[illegible]; × Phospholipid; ○ Haemoglobin.

after a considerable proportion of the membrane has been removed. This proportion appears to be more or less independent of the concentration of alumina.

The results suggest that the dissolution takes place in two steps. First, there is a rapid loss of superficial or surplus material which is not immediately harmful. Thereafter the rate of loss of membrane components is closely proportional to the rate of haemolysis. This suggests that any further loss of material from individual cells leads to their rapid and complete destruction.

Figs. 4 and 5 show the effect of temperature upon the rate of loss of lipids and the haemolysis of cells exposed to alumina. Between 40° and 5° the rate of haemolysis proceeds in a manner consistent with a process governed by simple diffusion, that is, with an activation energy of 5500 calories. Below +5° the rate of haemolysis and loss of lipoprotein begins to increase, but the loss of cholesterol and phospholipid continues to fall.

Previous experiments (Lovelock, 1954) indicated that the loss of components from the red cell is not reversible in a simple manner. Cells which have been denuded of membrane components regain very little of their lost material after one hour in fresh plasma at 37°.

The experiments just described were carried out using cells stored at 4° in "acid citrate dextrose" media for from 3 to 10 days. During this period of storage there is little or no change in the viability of the cells, as judged by their survival on transfusion (Loutit, Mollison and Young, 1943). It was noted, however, that the deleterious effects of both repeated washing and exposure to alumina increased during this period of storage.

The experimental evidence shows that a considerable proportion of the red cell membrane is easily detached by washing or by exposure to alumina. The proportion detached does not appear to be a defined superficial layer or capsule, since the proportion lost varies with the temperature and duration of cold storage. The removal of this layer is not immediately

harmful, and cells so treated do not haemolyse more rapidly than normal cells when suspended in 0·16 M-NaCl. The loss of components is, however, accompanied by a loss of volume.

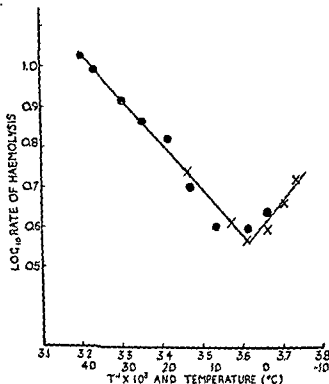


FIG. 5. The haemolysis of red cells in the presence of

This could result, for example, from changes in the internal composition of the cell due to alterations of its permeability. Recent preliminary measurement of the electrolyte and water

content of the red cell, after treatment with alumina, suggests that no alteration in the internal composition takes place. If, however, the lost volume is simply that which was occupied by the detached components, it is possible to calculate the concentration of these components in the detached layer. The results suggest that the concentration of the removable layer is equivalent to that of a 2 per cent lipoprotein gel. This agrees well with the value suggested by Mitchison (1953), based on birefringence measurements, for the concentration of lipoprotein in whole membrane. Ponder (1954), however, suggests that the membrane is much less hydrated than this and gives an estimate of 33 per cent for the concentration of membrane material from observations of the volume of fragmented ghosts.

It seems worth considering the possibility that the concentration of membrane components is not constant throughout its thickness but increases radially inwards from the surface. This could explain the ease of detachment of the tenuous surface layers, and to some extent resolve the discrepancies between the estimates of the membrane thickness and composition.

The classical view of the structure of the red blood cell membrane implies a framework of stroma protein surrounded by a bimolecular layer of lipids, with a sprinkling of antigenic protein at the surface. It seems unlikely that a cell possessing such a structure could lose a substantial part of its membrane and still remain intact. Among the recent views upon the architecture of the red blood cell, that of Moskowitz and Calvin (1952) agrees best with the experimental results above. They envisage a membrane composed principally of fibrils of a lipoprotein, elenin, orientated parallel to the cell surface and cemented together by ether-soluble lipids.

If the structure of the red cell membrane suggested by Moskowitz and Calvin is accepted, then the physical dissolution of red cells might proceed as follows: when the cells are suspended in a fresh saline medium the surface lipids will dissolve or disperse in the medium; the lipoprotein which was held in position by the lipids will then become detached and

diffuse away. In a medium maintained continuously unsaturated with respect to the lipid components of the cell membrane this process will continue until so much of the lipoprotein has unravelled that the intact existence of the cell is no longer possible. The increase in the rate of haemolysis below 5° could result from a hardening of the cementing lipids, if the repair of small breaches in the membrane depended on their ability to flow. Recent investigations of the effects of thermal shock on red cells suggest that such a hardening of the lipids may in fact occur (Lovelock, 1955*b*).

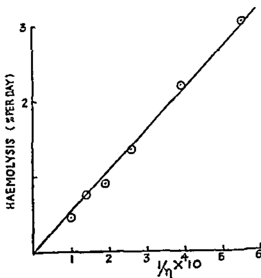
The free energy of the lipid components of a highly organized structure such as the red cell membrane is likely to be higher than that of the same components in their saturated solution. It follows that the red cell membrane is probably unstable in a physical sense, and its intact existence may well depend upon *the continuous synthesis of lipid components*, and upon the presence of a considerable reserve of membrane material.

The effects of cold storage

The experimental evidence so far shows that the red cell will disintegrate rapidly if kept in a medium which is not saturated with the components of the cell membrane. In their normal environment and during cold storage in their plasma the cells swim in the presence of a considerable excess of lipid and possibly other membrane components; in these circumstances it might be thought that damage by diffusion would not take place. The chemical analysis of red cells, after storage for a few months at 20°, indicated that changes very similar to those following exposure to alumina take place (Lovelock, 1954). Unless a physiologically abnormal medium is used for their storage, namely one which is acid and which contains a salt such as sodium citrate or lactate, a rapid dissolution of the cells takes place. The beneficial effects of the acid citrate medium used for the routine cold storage of red cells are directly attributable to its poor solvent action towards stroma lipoprotein.

Even in this medium, however, adverse changes do occur

and are made apparent by a progressive increase in the ease of detachment of membrane material by washing or alumina and by the ultimate failure of the cells to survive when transfused. These changes can occur at all temperatures between 0° and − 78° and are therefore presumably physical in nature, for below − 20° metabolism has effectively ceased. In a personal communication Dr. A. Richardson Jones reports that it is possible to produce a specific anti-serum for the human red cell envelope by immunizing rabbits with alumina powder after its exposure to red cells. The anti-serum has the remarkable property of haemolysing freshly drawn cells but not cells which have been exposed to alumina, repeatedly washed, or subjected to prolonged cold storage. The experimental result



The relationship between the rate of spon-

and 4·0 M.

strongly supports the notion that the changes in the cells during cold storage are similar to those induced by enforced diffusion.

If the dissolution of the cell during cold storage is preceded by the diffusion and dispersion of its membrane, it would be expected that the rate of dissolution would be inversely related to the viscosity of the medium and directly related to the solubility of the membrane material. Some confirmation of this is provided by the results shown in Figs. 6 and 7 where

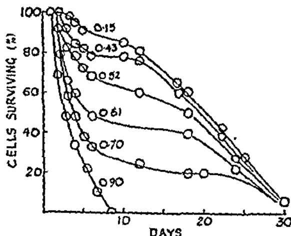


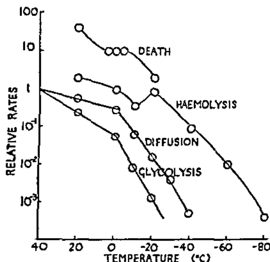
FIG. 7. The spontaneous haemolysis of red cells at 0° [illegible]

the spontaneous haemolysis of red cells in NaCl at 0° is seen to be inversely related to the viscosity of the medium and directly related to the NaCl concentration; the solubility of stroma lipoprotein bears a direct relationship to ionic strength. The effect of storage temperature on the rates of diffusion, metabolism, haemolysis and the death of the cells, as judged by their failure to survive when transfused, are shown in Fig. 8. The rates of glycolysis of the cells which are an index of their metabolism are taken from the data of Chaplin *et al.*

(1954), as are the death rates of the cells. The diffusion was calculated from the formula given by Einstein (1926),

$$D = \frac{RT}{6\pi\eta r}$$

where D = the diffusion rate, T the absolute temperature, R the gas constant, η the viscosity of the medium, and r the radius



tain the cells in liquid suspension.

of the diffusing molecule. For a given molecular species this relationship indicates that D is proportional to $\frac{T}{\eta}$. The relative rates of diffusion shown in the diagram are for the values appropriate to the glycerol concentrations and temperatures used. The viscosities of glycerol solutions at

temperatures below 0° are taken from the data of Miner and Dalton (1953) and of Blum and Kauzman (1954).

The diagram illustrates how very much more rapidly the rate of metabolism slows than does the diffusion when the temperature is lowered. At − 20°, assuming the relative rates are equivalent at + 40°, diffusion proceeds 20 times more rapidly than metabolism.

Both the rate of death of the cells and their spontaneous haemolysis show a cubic type of relationship with temperature. The rates of death and haemolysis decrease at first with falling temperature until in the region between + 5° and − 20° there is a halt in the death rate and an increase in the rate of haemolysis. At still lower temperatures both rates resume their decrease with decreasing temperature. At the lower temperatures both rates fall approximately as fast as the calculated rates of diffusion. In the region between − 20° and 8° metabolism proceeds at an appreciable rate and its beneficial effects may be responsible for the observed temporary decrease in the rates of death and haemolysis as the temperature rises.

More direct evidence in support of the notion that the structure of the red cell is in dynamic equilibrium comes from experiments on the transfusion of red cells after storage and other treatment *in vitro*. Burnett (1951) has shown that red cells, exposed to enzymes which modify their surface so that they are no longer able to absorb virus particles, are restored to their normal condition within a few days after their transfusion. Gabrio, Stevens and Finch (1954) have shown that red cells recover from damage suffered during cold storage after they are transfused into a recipient. They have also shown that the damage suffered during cold storage can to some extent be reversed *in vitro*, which suggests that the capacity for repair is possessed by the cell rather than the recipient. These experimental findings seem sufficient to justify the speculation that the factor determining the survival of the cell, either *in vivo* or after storage, is its ability to repair losses by diffusion or other changes. In addition, it would

seem not unnatural that those parts of the cell which organize the repair are themselves more resistant to the attrition of physical agencies than the components they repair. An interesting and somewhat paradoxical support for this notion comes from the experiments of Gabrio and Finch (1954). They find that the survival of red cells during storage at 4° [illegible] resistance to diffusion.

Conclusions

In accord with the best principles of serendipity this investigation has established some unsought facts on the structure and status of the red cell, but the goal, the solution of the problem of its senescence still lies ahead. Nevertheless, two conclusions which appear to be relevant to the general problem of ageing can be drawn from the experimental evidence. The first of these concerns cold storage; the use of cold storage as a method of suspending viability is now well established in practice and may increase in importance as an experimental technique for research on ageing. For example, the work of Billingham and Medawar (1952), and Billingham (1953) has shown that the reimplantation of cold-stored infant tissue into the same adult animal is technically possible. The experimental results suggest that the success of cold storage as a method of suspending viability results from the arrest of diffusion processes and not, as was commonly thought, from the cessation of metabolic activity alone. Should cold storage become established as a technique for the investigation of ageing processes, then the proper understanding of the mechanism of its action is necessary both for its theoretical implications, and for defining the optimum conditions of storage.

The experimental results also offer confirmation for the notion that the red cell is in fact a steady state system in

which active synthesis maintains the cell at a constant composition in spite of the dispersion and disorientation of its substance by random molecular movement.

It is possible to demonstrate physically that the [illegible]ence of a cell, so constructed, is [illegible] orm-ation [illegible] cell on t[illegible] [illegible]ysical structure, the interpretation [illegible] senescence in terms of physical dissolution remains speculative. In the subject of senescence, as in astronomy, the lack of essential facts is not usually a serious objection to the discussion of a theory. This precedent, and also because further experiments are suggested by the theory just outlined, seems to justify the discussion that follows.

It is at first sight perhaps surprising that a model cell, which is supplied with unlimited quantitites of energy and which has the means to repair damage suffered as a result of wear and tear, should be subject to inevitable dissolution. There are several ways of formulating this problem in physical terms, but the simplest of these makes use of "information theory" (Shannon and Weaver, 1949). Information theory is primarily concerned with the conveyance of messages. It is well established, in both the theory and practice of this subject, that it is impossible to convey information without some loss or distortion taking place, and this is entirely attributable to the universal tendency for disorganization to occur. Or in other words, to compose a message, something which is distributed at random must be organized and this organization increases its content of energy. The normal and inevitable degradation of this energy with the passage of time results in an equivalent degradation of its content of information.

In order to preserve its identity in the face of wear and tear, a living cell must possess at its formation a considerable quantity of information to direct the course of its [illegible] functions. Whatever part of the [illegible] is itself [illegible] move[illegible] [illegible] tion. [illegible] open to the criticism that it

states the obvious in different words. Nevertheless it has the advantage of directing attention to that part of the cell which is most vulnerable to the effects of physical dissolution, namely the part which conveys its specification and which alone cannot be repaired if damaged. Furthermore, it would suggest that even the non-nucleated mammalian red cell possesses all the information necessary to preserve its identity during its life-span. This suggestion is in accord with the observed ability of the cell to repair itself after damage during cold storage, and by the constancy of its composition in its normal environment.

Finally, there is an analogy which illustrates the relevance of this particular physical approach to the general problem of ageing. In the practice of information theory, which is called communications engineering, there is a device for rejuvenating messages which would otherwise have become unintelligible during their transmission over a great distance. This "ageing" of messages can be overcome if the same message is received simultaneously over several different, but closely adjacent, paths and then combined. The probability of losing the same items of information along each of the paths is small, and so the combination is usually a faithful replica of the original. There seems to be more than a casual resemblance between this device and the natural process of conjugation.

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[illegible] *Theory of* [illegible]

[Discussion of this paper was postponed until after the paper by Dr. Mollison.—Ed.]

AGEING IN HUMAN RED CELLS

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AGEING in red cells suggests at least three processes, all of which may be quite unrelated. First, there is the transition from characteristics displayed by red cells in infancy to those displayed by the same cells in adult life; secondly, there are changes occurring during the life-span of individual red cells; and thirdly, there are changes which occur when red cells are stored outside the body.

It has been shown that the red cells of newborn infants differ in several respects from those of adults; for example, they are larger and contain a considerable proportion of foetal haemoglobin. It seems probable that the characteristics of the cells of newborn infants reflect changes during the evolution of the species or, in some cases, represent adaptations to foetal life; for example, the weakness of the A antigen at birth may help to protect the group A infants against the A antibody which may cross the placenta from its mother's circulation. A true age change observed in red cells

the kind occurring within the life-span of a given population of red cells.

The life-span of red cells in many species has been extensively studied in the past 20 years. The most direct evidence has been obtained from experiments in which red cells formed during a limited period of time are labelled. For example, if radioactive iron is injected into dogs the iron is incorporated into newly formed haemoglobin. Provided that the animal is given an adequate amount of non-radioactive iron, radioactive

iron liberated from breaking-down red cells is not reutilized, and so the disappearance of cells from the circulation is accompanied by a disappearance of radioactivity. Such experiments show that most red cells in the dog live for about 110 days; they also show that the standard deviation of life-span is quite small—about 6 days (Brown and Eadie, 1953).

When red cells are transfused from one normal subject to another, a very different type of curve is found because the blood then contains labelled red cells of all ages. Since red cells live for rather more than 100 days, each day slightly less than 1 per cent of the cells are expected to reach the end of their life-span and leave the circulation. Transfusion experiments lend solid support to the idea that red cells have a definite life-span which, in man, is about 110–120 days.

Dornhorst (1950) has aptly compared the red cell to the V-1 pilotless bomb, starting on its journey with a definite load of fuel and crashing precipitously to earth when the fuel is exhausted.

At one time it was suggested that red cells were slowly battered to pieces in the circulation (Rous and Robertson, 1917). However, this view is more in keeping with the idea that red cells are inanimate particles rather than that they are actively metabolizing cells. There is some direct evidence against the idea that cells are battered to pieces after a finite number of collisions in the circulation. For example, if red cells are transfused to a patient with double the normal cardiac output so that they travel faster and further than normal red cells, they survive for the same time as in a normal circulation (unpublished observations).

In fact, it appears that red cells are capable of a limited amount of self-repair; for example, they can synthesize phospholipids and thus replace lipids lost from their surface (Altman, 1953). It is of some interest that red cells which have been washed a dozen or more times in normal saline, and have presumably lost some cell lipids, survive normally after re-injection into the circulation (Hughes Jones and Mollison, 1956).

It seems probable that what red cells cannot do is to synthesize enzymes. It is known that the cholinesterase activity of reticulocytes is two to three times higher than that of mature cells (Sabine, 1951). It has also been shown that the cholinesterase activity of rat red cells increases following haemorrhage and then gradually declines (Pritchard, 1949). Recently, Allison and Burn (1955) have shown that the cholinesterase and catalase content of transfused red cells diminishes progressively with time.

It thus seems very likely that the fuel of the red cell which becomes exhausted with time is its content of enzymes. When the enzyme content falls to a certain level, the active processes upon which the integrity of the red cell depends cease and the red cell disintegrates. It is known that the red cell derives most of its energy from anaerobic glycolysis. However, *it is not known how failure of the supply of energy* determines the death of the red cell.

One possible experimental approach to this problem would be to treat red cells with various agents which might enhance or destroy a particular enzyme system, and then study the effect on the survival *in vivo* of the treated cells. This possibility is suggested by the observation that when red cells are treated with more than a certain amount of sodium chromate their survival is interfered with. When a certain dose is added the red cells at first survive normally, but then after about 14 days in the circulation the death rate suddenly rises and within 10 days almost all are gone (Hughes Jones and Mollison, 1956). A study of the way in which this premature ageing of red cells is brought about might be very rewarding. It is possible that the damage is not due directly to chromium but rather to oxidation. When sodium chromate is added to red cells mixed with A.C.D. in amounts of the order of 50 μg./ml., sufficient methaemoglobin is formed to turn the suspension of red cells brown. Although the presence of methaemoglobin in red cells in congenital methaemoglobinaemia does not alter their life-span (Hurley and Weisman, 1954), it is probable that the addition of chromate oxidizes other substances in the red

cells besides haemoglobin and in this way may cause irreversible damage.

Although in principle it is possible to obtain samples of young and old red cells separately, comparatively little work has yet been done upon differences between them. A population of very young red cells can be obtained by repeatedly bleeding an animal, or by allowing red cells to sediment *in vitro* and separating the more slowly sedimenting cells. A population of old red cells can be obtained from an animal in which erythropoiesis has been temporarily suppressed.

One approach is to label a population of young cells with radioactive iron, and then to take samples at intervals during the life-span of the labelled cells. It is comparatively easy to discover whether the labelled cells differ from the recipient's own cells in various respects. For example, blood samples can be mixed with a series of hypotonic saline solutions and it can be determined whether an undue proportion of old cells have been haemolyzed, judged by radioactivity liberated from labelled cells. Using such a method Cruz, Hahn, Bale and Balfour (1941) showed that old red cells are more resistant to hypotonic haemolysis. Stewart *et al.* (1950) confirmed this and showed that old red cells were less resistant to mechanical trauma.

Because so little is known about age changes in red cells *in vivo*, it is worth considering changes occurring on storage *in vitro* and discussing whether these throw any light on the normal ageing process.

If blood is mixed with citrate and stored at + 4° C the red cells slowly deteriorate. "Deterioration" is used here to mean a loss of the ability of the red cells to survive normally in the circulation. The rate of deterioration can be slowed by the addition of dextrose (Rous and Turner, 1916), or by acidification (Loutit, Mollison and Young, 1943). The rate of deterioration can be virtually arrested by storage in the frozen state. For example, red cells stored at − 79° C for periods up to two years show no progressive loss of viability with time, apart from a small loss observed after a few weeks' storage (Chaplin,

Crawford, Cutbush and Mollison, 1956). Notice that no ageing *in the ordinary sense has occurred since such cells are still* capable of surviving for three months after transfusion. Even when red cells are stored at $-20°$ C the rate of deterioration becomes very slow after the first six months (Crawford, Cutbush and Mollison, 1955).

Many of the changes that occur in red cells during storage are reversible *in vivo*. For example, alterations in osmotic fragility evidently depend to a large extent on the electrolyte content of the storage medium. At $+4°$ C the normal active cation transport across the red cell membrane is virtually arrested and thus there is a slow shift towards osmotic equilibrium. In media containing citrate this may lead to a considerable loss of base from the red cells. These changes are reversed after transfusion although it may take more than four days for the reversal to be completed (Crawford and Mollison, 1955).

It has been shown that there is a rough parallel between the decrease in ATP content of red cells and post-transfusion survival (Rapoport, 1947). Finch and his co-workers (Gabrio, Donohue and Finch, 1955) have shown that when adenosine is added to blood, the ATP content of the cells is better maintained and viability is retained for a longer period.

However there is considerable evidence that deterioration during storage is very different from ageing *in vivo*. For example, when red cells stored in A.C.D. at $+4°$ C for two weeks are transfused it is found that about 10 per cent are removed from the circulation within a few hours and the remainder survive as well as *fresh red cells. Note that it* cannot be the oldest red cells which have been lost since the survival curve cuts the time axis at the same point as the curve for fresh red cells (see Mollison, 1951).

If red cells stored for three weeks are transfused to a subject, then removed from the circulation, stored for three weeks and again used for transfusion, they survive in the same way on the second occasion (Gabrio, Stevens and Finch, 1954).

Evidently, then, ageing *in vivo* and deterioration at $+4°$ C

are different processes. It has already been mentioned that changes in the sodium and potassium content of red cells during storage are reversible after transfusion. It has been shown that changes in the content of organic phosphate compounds are reversed within three hours of transfusion but are not reversed by incubation for 24 hours *in vitro* with fresh plasma (Gabrio *et al.*, 1954).

It is possible that ATP plays an important rôle in the maintenance of viability of normal red cells. It has, for example, been shown that in hereditary spherocytosis, in which the life-span of red cells is greatly reduced, the rate of ATP formation is diminished (Prankerd, Altman and Young, 1954). It is also known that reticulocytes contain two or three times as much ATP as mature red cells (Granick, 1949). However, work in this field is still in an early stage.

All that we can say at the present time is that ageing of red cells almost certainly consists in the gradual using up of an initial supply of "fuel" that cannot be replaced, and that changes occurring in red cells during storage probably tell us very little about ageing *in vivo*.

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DISCUSSION

Krohn: I have got three questions altogether; two for Dr. Mollison. The first is: do the red cells of infants and children or young animals age or have a different life-span from the mature cells of the adult person?

The second one was: in the slide you showed of the chromium-treated red cells, the cells seemed to be normal for some length of time and then suddenly were destroyed; is it possible that your treatment with chromium altered the proteins of the cells so that they became immunologically incompatible and that this sudden removal of the cells was the development of an immunity reaction so many days after their introduction?

And the final question, for Dr Parkes, was that I wondered whether the choice of red cells was very convenient? They have not got any nuclei and they are not really very good cells in a sense, I should have thought to work on.

chemist is its sharp end-point in haemolysis.

Mollison: I was not quite clear what you meant by "not good for ageing experiments", Dr. Krohn. In what sense?

Krohn: It is so atypical a cell that it seems to me you want a cell which has got a nucleus inside it. A lot of these changes that take place in the red cell may depend on the fact that it has not a got nucleus.

Mollison: Yes, but it does seem to have a sufficiently complex metabolism; it may be better to start and understand it and then go on to a more complicated cell.

Rowlands: I suppose the other possibility would be to use avian blood cells.

Montagna: Perhaps, but suppose one were to take Dr. Krohn's suggestion and use a suspension of epidermal cells, they might do a

demonstrate thermal shock?

Parkes: Yes, that is true.

Krohn: Is that again a possibility that thermal shock depends on the presence of the nucleus?

Parkes: I do not know about that. It shows thermal shock only in abnormal media—in hypertonic media.

Huggett: Are there any essential points of fundamental difference between the mammalian non-nucleated red cell and the avian nucleated red cell, Dr. Mollison?

Mollison: I think one very important point is that the non-nucleated

Mollison: I do not know. I know only that it has been shown by Maizels that in nucleated red cells cation transport depends upon

synthesize enzymes.

Amoroso: But then you say that reticulocytes have a greater capacity to do this?

Mollison: Yes, but once the reticulocyte stage is over, apparently they cannot do it any more.

red cells?

Mollison: It is merely that they synthesize haem while they still have got some reticulum, for example, and once they have lost this they cannot synthesize haem any more.

Amoroso: So the reticulum is important in respect of that particular property?

Mollison: Yes.

long time.

A nucleus may then be transplanted back into that enucleated cell and

show holes in the red cell membrane.

micrographs.

While we are on that point, there is one other thing and that is that Dr. Mollison showed a slide of the effects of repeated washing, and

Lovelock had the same kind of result, in that to start with there was a rapid loss of lipoprotein from the cell without anything very much

a loss of lipids is important. Lovelock, I think, was using fresh cells in the experiment quoted. When he dealt with stored cells, he found much greater loss of lipids. We have recently done some experiments where we have seven times washed cells stored for about five weeks and we have obtained just the results we expected with unwashed cells. So I still do not know how important this loss of lipids is in practice. Similarly, in some of the cases of ours in which Lovelock actually estimated lipids on the stored cells, we sometimes found poor survival when there was very little loss of lipids. So that although of course I do not doubt the fact that red cells do lose lipids, I am not sure

phate is a surprising thing for a dead cell to do?

stem

orry,

one

seems to be perennial.

ence it has lost its reticulum it does

in; in fact I do not think it can

because its life-span can be estimated from the incorporation of amino acids into the protein part of the globin.

because work in a certain type of experimentation is applicable to the living cell the biophysical system in that experimental technique is living too? I think what it really comes to is that Lovelock only claimed to have a very useful biophysical system to work on.

Parkes: That is exactly what it does mean.

Huggett: It does not deny Williams' point, that it may be dead in the biological sense of the word.

Villee: I think we could settle this only if someone can give a definition of life which is acceptable to all of us and then we might decide whether the red cell does or does not fit that definition.

Dempsey: I thought we were having that trouble with the concept of ageing!

Amoroso: Is there any evidence that nerve cells grow and multiply?

Krohn: Grow, but not multiply.

Amoroso: Are they alive, according to Williams, or are they dead or only partially alive?

Williams: I should think they have synthetic activities.

Wislocki: With respect to the ageing of cells, I find the question

theless, at the molecular and atomic level of structure, all cells and interstitial substances appear to be repeatedly renewed. The nature

Parkes: May I ask Dr. Mollison, when the red cell is about to fade

which has emerged from recent work at Oxford, is that it looks as though its content of enzymes is getting low, but that evidence is rather incomplete at the moment.

Huggett: Mollison, you said a few minutes ago that the red cell has an anaerobic metabolism. If you have fresh blood, its cell reduces. Where

on it?

Mollison: I do not think so; I think the main thing is anaerobic glycolysis; red cells use dextrose quite rapidly.

Parkes: I gathered from what you said, Dr. Mollison, that in your later experiments you have got the red cell apparently stable at low temperature. Is that right?

Mollison: It looks like that; of course, it is for periods only of the order of one year.

Parkes: Two years, I thought.

[illegible] onths [illegible] loss, [illegible]

there is an early period of fairly rapid deterioration, followed by only very slow deterioration, I cannot believe random molecular movement is the chief or only factor responsible.

Parkes: Not as compared with diffusion.

Mollison: No. We do not understand at all well what is happening; at $-20°$ C, for example, we do not understand why there should be a progressive drop for three months and then, apparently, so little change afterwards.

Parkes: How then do you explain the fact that altering the medium has stopped the slight but steady loss which previously occurred at $-20°$ C?

Mollison: I think the red cells were bursting. We mixed them with saline glycerol and thus there was, inside the cell, haemoglobin exerting an osmotic effect, and outside, effectively nothing. Now we use citrate which is a non-penetrating anion outside, which acts as a balance to the haemoglobin. We find that the cells actually shrink in this medium. I think in the other medium they were simply bursting.

Parkes: They were swelling up at low temperature?

Mollison: Oh yes. We know that potassium and sodium shifts across the red cell membrane go on quite rapidly at $-20°$ C and occur even at $-79°$ C.

Parkes: Of course, that is just another form of physical dissolution at low temperature, is it not? Physical instability?

Mollison: Yes, it is physical instability but not dissolution.

[illegible]

Mollison: No.

T.-Duplessis: I just wanted to ask you if there is some relationship in the life span of a red cell from an animal with a high metabolic rate [illegible] span of the red cell?

Mollison: I do not think I can be very useful on that. I showed the dog as having a red cell life span roughly the same as in man. Rabbits [illegible] life-span—100–120 days.

Villee: It might also be interesting to study red cells of a hibernating animal before, during and after hibernation.

Mollison: Yes. In the last year or two somebody studied red cell life-span in the hibernating animal. I believe the results were published in *Blood* last year.

Parkes: I think when you visit our laboratory, my colleagues will be able to tell you something about the effect on the red cells of freezing the whole animal.

GENERAL DISCUSSION

Amoroso: We now come to the group discussion on the significance and limitations of the work discussed earlier from the aspect of

him to open the discussion?

Huggett: I wondered whether we could perhaps start at the beginning with simple definitions and see whether we can agree on those and then afterwards we could amplify into more general interchange.

Amoroso: I should have thought that in the course of our deliberations we have used the word ageing in every one of its possible connotations, so that I am a little doubtful whether we can define it more precisely than Dr. Parkes when he says it is the changing chronological events in the life history of the individual.

Corner: Is it not necessary to put the word 'irreversible' into Parkes' definition? If my arms are extended one moment and retracted the next, that is a change with time but it is not ageing. I am suggesting that in your definition that ageing is a change of

time change.

sudden t fertilization?

Wisl hich it is used, including growth, differentiation, maturation and senescence. For many purposes, however, the term ageing is better defined as senescence, by

which is meant a process of unfavourable, regressive, terminal changes which ensue after maturity (Lansing. Problems of Ageing, The Williams and Wilkins Co., Baltimore, 1952). *Ageing*, as thus defined, is inversely related to growth and maturation and involves, instead, a decrease in efficiency of the mechanism for reconstruction.

time?

functions. In growth we have got greater functional usage and that

phorus.

Amoroso: It is possible, however, to maintain speculative doubt whether the changes of senescence are wholly inherent and inevitable in the course of life. In the first place there is, as Professor Montagna remarked this morning, considerable variability in the time of onset of these changes. Secondly, in certain forms of life "ageing", as

but also fresh water fish.

ou have the
orget that if
vertebrates,

both fishes and snakes. Warm-blooded and cold-blooded ... thus *do not age in the same way.*

Wislocki: It certainly is important, in order to achieve clarity, to define what one means by ageing. With respect to the placenta, which I discussed yesterday, I distinguished between its growth and differentiation, which represent the initial phases of ageing, and of senescence which is the terminal phase. The latter, furthermore, which is regressive, may be either normal or pathological. I also raised the question of the difficulties of ascertaining the degree of senescence which the placenta actually undergoes.

[illegible]

growth, senescence begins?

Bourlière: May I remind you that Prof. McCay's experiments, started more than twenty years ago, have beautifully shown that growth and ageing processes, although somewhat opposed, are nevertheless mutually interdependent? At least, in cold-blooded vertebrates it is now quite obvious that when one slows down the growth rate, one increases the life-span. In other words, the quicker the growth of a poikilothermous vertebrate the shorter its life-span.

Montagna: In McCay's experiments, on the other hand, many factors complicate the picture; for instance, these animals are not sexually mature and remain infantile. Who knows how many other [illegible]

hamster—as I believe he can do now for some weeks—does that add to its life-span?

Parkes: It is a shallow-freeze for only an hour or so.

Wislocki: I see. However, do you know, if a hamster were subjected to extensive periods of ordinary hibernation at a low temperature (as could be arranged experimentally) whether the time spent in hibernation would be directly added to its life-span and hence modify its ageing?

[illegible]

that *Rhinolophus ferrum-equinum* and *Miniopterus schreibersi* could reach at least 14 and 15 years!

Wislocki: Then you believe that one can prolong the life expectancy of a mammal by reducing the metabolism?

Bourlière: I think so.

Krohn: Have attempts been made to keep animals, that normally hibernate, out of hibernation during each ordinary hibernating period? Would that shorten their life-span?

Bourlière: As far as I know that experiment has not been done.

Krohn: That would be relatively easy to do.

Parkes: The trouble is, I think, that not enough is known about the hibernating mammals to know what their life-span would be without hibernation.

Krohn: We know little about the natural life-span of any animals, come to that.

Wislocki: It could, perhaps, be fairly readily found out with a colony of hamsters.

Parkes: I suppose, yes, if they could be frozen for a significant time.

Wislocki: If one group could be induced to hibernate to the maximum degree during their lifetime and another group kept

and ceases to breed in the winter but it does not go into a proper hibernation. I can see technical difficulties there.

Williams: It does not necessarily stop breeding either, I think. Certainly in some of our colony we have been selecting those for winter breeding; you can do that.

Yemm: It seems to me that there is some difficulty in terminology and that we should make the term 'ageing' apply to the life-span of

useful discipline to reserve the term 'ageing' for the organism as a whole, as a chronological relation in its development and ontogeny; and 'senescence' as a term referring to the physiological condition of its different tissues or organs.

Villee: I think that is a very good point. Prof. Montagna's figures

this morning showed how with cells lying side by side in the same orga[illegible] in one and [illegible]

K[illegible] a local process, not a general process [illegible] now, the [illegible] he time [illegible] of the individual; probably muscle does the same. In contrast to that, there are many tissues in which there is a continuous growth and continuous destruction of the cells going on simultaneously, so in one organism you can have a cell, the age of which is equivalent to the chronological age of the individual, or conversely you can have a young cell in a young person or an old cell in a young person or a young cell in an old person, in fact, all the combinations and permutations.

Villee: This implies that you can date a cell from the time of the last cell-division, that it is then a new cell, and I wonder if you really mean to imply that?

Dempsey: Well, I think you have to have some data to operate with and it seems to me that the two possible phenomena that you can use for dating are: (1) the last previous mitosis and (2) the last previous fertilization.

Villee: Yes, those are two useful ones.

Dempsey: But are there any others?

Villee: Well, I am not at all sure that you can set up, other than arbitrarily, some point at which to speak of a new cell. Certainly the protein and other compounds in the cell are constantly being renewed, but I do not think there is any time when they start completely afresh.

Dempsey: But I am thinking of the fact that, in tissue cultures, most cells are potentially immortal and that they continue to divide and, therefore, so long as the process of division is maintained the cell remains viable and does not become senescent, whereas post-mitotically the cell does then begin to age and become senescent and ultimately dies.

Mitosis is rather orderly in the intestinal epithelium in that it occurs at the base of the villi, and the older cells occur at the tips of the villi, and there is an orderly progression.

Fawcett: Is not one of the best examples to be found in the cartilage columns of the epiphyseal plate? There you have a row of chondrocytes of all ages. At one end are cells that have just divided, then a series of cells in various stages of maturation, and finally, at the other end, are cells that are ageing and dying. It is essential

[illegible] is accelerated and this results in a premature [illegible]

[illegible] a slightly different con- [illegible] as the accumulation of [illegible]

development? In other words, can you get an amitosis; how much does it exist; how much does it come into the picture?

[illegible] references in the literature to ami- [illegible] omes do not form. [illegible] normal cells ever [illegible] duc, E. H. (1948). *Amer. J. Anat.*, 82, 353).

Huggett: I raised this [illegible] and I have been looking at cuttings [illegible] ow, though the whale foetus [illegible] the primate foetus *in utero*, [illegible] it may be we have not sampled early enough; but for the moment, it looks as if the simplest thing about this fast-growing organism is [illegible] absence of mitosis.

[illegible] no index [illegible] he *tissues* [illegible] l been re- [illegible] itosis had [illegible] fact that [illegible]

[illegible] liferative zone, or matrix, is very small. As the cells move up from the matrix they increase many hundreds times in volume; this is a very important factor in growth. Hollander found that after he

washed a dog's stomach with eugenol, which completely denuded the mucosa of the stomach, the mucosa was repaired within 24 hours. This provisional repair takes place without the occurrence of mitosis. The cells of the foveolae and the neck-chief cells simply expand; they glide over and very quickly repair the denuded stomach. Mitotic activity comes much later, after the damage has been largely covered over, and final repair of the damage takes place.

As far as amitosis is concerned, I rather suspect that there is no such thing.

Huggett: I should not be the least bit surprised.

Jost: I should like to return to embryonic problems. I feel that ageing of a tissue involves a very important period during which the potentialities of the cells are limited—what the embryologists call "determination of a tissue". The time at which cells become specialized for a special function is certainly a great event in a tissue; I believe that in ageing of a tissue this is an important part and a first step towards senescence.

I hope that the biochemists will define "senescence". Perhaps the difference between synthetic and catabolic activities, as we were told this morning for leaves, may be used.

Williams: I do not want to take that point up but can we perhaps think that senescence is a quality correlated with sexually reproduced [illegible] is intri[illegible] tively [illegible] I can [illegible] reproduced is the transplanted tumour which is also intrinsically immortal.

Corner: Jennings, who made a considerable point of senescence not in the individual protozoan, e.g. *Paramecium*, but in the clone (the totality of cells derived from a single mating between two *Paramecia*), said that the offspring of a pair forms a kind of unit which undergoes senescence in that its cells finally lose the power to reproduce by division and the clone dies out unless one of them mates again.

I think he had a poetic notion that the clone of *Paramecium* is like the multicellular body of a higher organism; in that sense the [illegible]

Krohn: Sonneborn has been working on this recently, has he not?

He relates the ageing of the clone to interference with the normal division of the micronucleus; once that starts to go wrong the strain dies out.

Dempsey: I think there are some species of invertebrates which reproduce parthogenetically and in which sexual stages never have been observed.

[illegible] began.

* * * * * *

[illegible] cussions. In doing so, I shall indicate to you to what extent my education on the ageing of transient tissues has been improved and to what extent hiatuses still remain which must needs be filled. If, therefore, any part of this summary fails in its allusion to major points whilst emphasizing minor ones unduly, please ascribe this not to any discourtesy but rather to my own limitations.

I shall first take advantage of having the last word by defending myself in respect to not providing a definition of ageing in my opening remarks. To anyone who has listened to our several dis- [illegible] straightforward statement [illegible] used [illegible] s, yet we have, none of us, succeeded in formulating a simple definition to cover all the facts.

Having thus defended my own rear, I pass on to summarize the contributions.

[illegible] suggested by Dr. Price, Professor [illegible] sider the effects of ovariectomy in genetic females is, however, to be regretted, since such procedures might well have provided information on the much disputed question of the manner of growth of the

Müllerian ducts. In mammals, the Müllerian system is derived from both pronephric and mesonephric rudiments, whilst in elasmobranch fishes it arises by longitudinal splitting of the Wolffian ducts. Consequently, the part played by the ovary, in furthering Müllerian differentiation whilst suppressing Wolffian development, might repay further study. Furthermore, the employment of the methods of tissue culture, using synthetic media, should as Dr. Price rightly emphasizes, greatly advance the study of ageing and differentiation.

Jost gave numerous data on the effects of decapitation on mammalian foetuses, especially rabbits and rats. In particular he called attention to the importance of the pituitary for testicular development and for controlling liver glycogen. The stepwise utilization of hormones by the growing foetuses, as well as the limited period of action of the foetal endocrines struck me as points of particular interest. Perhaps the manner of utilization of hormones are necessary evolutionary steps and their abolition would prevent evolution. However, while it is impossible to deny the importance of the foetal endocrines, I am convinced that their limited period of action and the further fact that they act during periods of great sensitivity of the target organs, are in some way implicated with the endocrine functions of the placenta.

[illegible] biol [illegible] alw [illegible] the [illegible] for a moment minimize the importance of genetic factors in the causation of congenital abnormalities, it must be realised that developmental processes can be altered by environmental disturbances as well as by abnormal genes. Thus Jost demonstrated certain teratological effects in rats which result from the administration of pitressin and cortisone; pitressin producing amputations if given before the sixteenth day of pregnancy and cortisone producing cleft palate. I do not believe that these are specific effects, nor should [illegible]

[illegible] the twenty-fifth day of pregnancy, for as Prof. Wislocki reminds us Claude Bernard was the first to propose that the placenta was a deputy for the foetal liver, until such time as the latter was physiologically capable of forming and storing glycogen. Consequently, since glycogen storage in rabbit foetal liver may be postponed by decapitation, it would be pertinent to know whether the placenta still retained its glycogen storage functions when the foetal liver was so influenced.

[illegible]

demonstration that growth hormone inhibited foetal growth only *during the period of the normal gestation time* and that thereafter the embryos grew at an accelerated rate. We may infer, therefore, that the rabbit placenta which is commonly regarded as havir [illegible] y-first day of pregr [illegible] fficiently and with [illegible] criteria the placenta can scarcely be regarded as senescent.

Zuckerman, Williams and Rowlands dealt with different aspects of ageing in the transient tissues of the ovary. Zuckerman boldly and appropriately opened with a discussion of the germinal epithelium. He argued that the regenerative capacity of the germ cells was poorly developed, and the ten points which he advanced in [illegible]

[illegible]

problem of ageing of transient tissues as can at present be made. Moreover, Rowlands' observation of the inability of induced corpora lutea to prolong pregnancy in the guinea pig merits further investigation, since induced corpora lutea have long been regarded as a reliable means of extending the duration of pregnancy in some animals. It is possible that the time of induction may be the *deciding factor.*

[illegible] on, [illegible] and [illegible] ore [illegible] ere associated with profound changes in morphology and biochemical stability. However, the results that have been presented can hardly be said to support this view, for as judged by its biochemical activities and by its structural characteristics at term, the placenta [illegible]

[illegible]

osis. In each case the electron microscope has been used to provide the most elegant series of electron micrographs that it has been my privilege to see. Their studies are as valid a contribution to the study

of cellular differentiation as is Wislocki's contribution to the growth cycle of deer antlers. When a dozen studies of similar amplitude [illegible] have a well defined branch of geronto-

[illegible]

ageing in transient tissues as can at present be made.

Montagna's paper was useful in presenting results derived from the human female in circumstances which permitted a freer and cooler discussion than is often possible. His was an extensive analysis of a problem complementary to the intensive analysis of the red blood cells by Lovelock and Mollison.

Evidence from botany was presented by Yemm. I had hoped that he would have told us why monocarpic plants mature seeds but once and die. This he did not do. Nevertheless, his view that even in senescent leaves there is an appreciable amount of synthesis and respiratory activity, suggests that ageing in plants may be a very laggard process indeed.

Drawing these various lines of research together, I feel embar- [illegible]

like the ageing process itself, must advance in steps and I feel that this symposium has made a definite contribution to the solution of the problem of ageing.

It is true, as Professor Zuckerman once said, that genius, luck, fashion and expediency always seem to underlie man's greatest achievements. Luck, yes, but having heard Dr. Parkes' remarks I am sure that "Fortune only favours the prepared mind".

The coming years will bring new observations and insights, be it in the directions indicated above, or along other lines. In some years hence we may again be wondering where we stand and what progress we have made; let us hope that we may meet again at the Ciba Foundation in London.

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